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PRINCIPAL INVESTIGATOR: Sandra J. Gendler, Ph.D.

CONTRACTING ORGANIZATION: Mayo Clinic Scottsdale
Scottsdale, AZ 85259

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13. ABSTRACT (Maximum 200 Words)

This project is focused on novel tumor vaccines directed at MUC1 and other tumor antigens. Our specific aims are: 1)To assess the effectiveness of vaccines against MUC1 and other tumor antigens in the prevention and treatment of spontaneous breast carcinomas in mice; 2)To translate an effective vaccine strategy into a phase I clinical trial in patients with undetectable disease following standard therapy. The model of spontaneous mammary cancer is the MUC1-expressing polyoma middle T antigen mice (MMT). We have tested five vaccines in the preclinical mouse model: 1)liposomal MUC1 tandem repeat peptide, 2)dendritic cells (DCs) pulsed with tumor lysate, 3)DCs fused to MMT tumor cells, 4)adoptive transfer of MUC1-specific cytotoxic T lymphocytes (CTLs)with CD137 co-stimulation, and 5)MUC1 peptides with CpG ODN and GM-CSF as adjuvants. All vaccines elicited a strong immunological response. Adoptive transfer of MUC1-specific CTLs with co-stimulation (CD137 mAb) significantly reduced tumor burden and tolerance in MMT mice. The peptide vaccine prevented MUC1-expressing tumor growth. The clinical trial is in the final stages of review. It is a phase I trial testing MUC1 and HER-2/neu class I and class II peptides with CpG ODN and GM-CSF adjuvants in breast cancer patients free of disease.

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INTRODUCTION

This project is focused on the development of novel tumor vaccines directed at MUC1, a transmembrane mucin that is aberrantly expressed in cancer. MUC1 is expressed on greater than 90% of breast cancers and often elicits cellular and humoral immune responses in humans. However, these responses are not sufficiently strong to eradicate tumors. MUC1 is a candidate peptide for novel immunotherapy strategies to strongly activate the immune system to eradicate tumors expressing these epitopes. In tumors, there is strong over expression of MUC1 on tumor cells and in circulation, expression is no longer restricted to the apical domain of cells, and glycosylation is altered, revealing immunodominant tumor-specific peptide sequences.

In our preclinical studies we have utilized mice that develop spontaneous mammary gland cancer that express MUC1. MUC1 transgenic mice (MUC1.Tg) were bred with mice carrying the MMTV-driven polyoma middle T antigen (MT) to create MMT mice [1, 2]. Mice transgenic for this protein develop B and T cell tolerance and are refractory to immunization with the protein encoded by the transgene. All mice are congenic on the C57BL/6 background to eliminate strain-specific modifier effects. In the MMT mice, mammary gland tumors are induced by the action of a potent tyrosine kinase activity associated with the polyoma virus middle T antigen driven by the mouse mammary tumor virus long terminal repeat (MMTV) [2]. Middle T specifically associates with and activates the tyrosine kinase activity of a number of c-src family members, eliciting tumors when a threshold level of gene product has been attained. This promoter is transcriptionally active throughout all stages of mammary gland development and results in widespread transformation of the mammary epithelium and the rapid production of multifocal mammary adenocarcinomas in 100% of the female mice. The MMT mouse appears to be an appropriate model for human cancer and allows us to study the effects of self-tolerance, immunity and auto-immunity to MUC1 as mammary tumors develop spontaneously [3].

The **hypothesis** of our study is that enhancing MUC1-specific immunity will result in anti-tumor immunity. We propose to develop an optimal cancer vaccine using epithelial cell mucin MUC1 peptides or protein or MUC1-expressing tumors presented by DCs as immunogen. The most successful therapies will be tested in a phase I clinical trial. An additional hypothesis is that tolerance occurs within the tumor environment, although immunization strategies can be developed to overcome tolerance.

Our **specific aims** are: **1)** to assess the effectiveness of vaccine formulations against MUC1 in the prevention and treatment of spontaneous breast carcinomas in mice and **2)** to translate an effective vaccine strategy into a Phase I clinical trial in patients with minimal residual disease. The draft clinical trial protocol for aim 2 is included in this annual report, although it has not yet received IRB or FDA approval nor been formally submitted for USAMRMC approval. These approval processes are underway.

RESULTS (BODY) (Figures at end of Results section)

Specific Aim 1: To assess the effectiveness of vaccine formulations against MUC1 and other tumor antigens in the prevention and treatment of spontaneous breast carcinomas in mice.

Our preclinical studies were to have been completed at the end of year two. However, we had several studies underway, which were completed during year three. These will be described below.

Tumor Escape Mechanisms A major problem with immunotherapy is that it is often administered to patients with large tumor burden who are well known to have defective functioning of the immune system and many tumor escape mechanisms in place [4, 5]. The challenge is to overcome these tumor escape mechanisms and achieve effective results in the spontaneous mammary tumor MMT mice and, most importantly, in patients with breast cancer. Tolerance is brought about by failure of the T cells to efficiently expand to antigen, and by the surviving cells entering into a state of hypo-responsiveness to subsequent antigen encounter. Although tolerance minimizes autoreactivity, it represents a serious problem in

diseases such as cancer, where a lack of a T cell response can prevent immunity. Co-stimulatory receptors are known to prevent tolerance induction before it has fully developed and hence they are promising targets for therapeutic treatment. The MMT tumors exhibited several escape mechanisms that are well documented in humans. They down regulated expression of class I molecules on the tumor cells and produced a number of immunosuppressive factors, including TGF-β2, IL-10, Cox-2 and PGE$_2$, all of which may render the CTLs cytolytically anergic (Fig. 1) [3].

CTL Homing and Tolerization

To test whether MUC1-specific CTLs enter the mammary tumor bed and are active within the tumor environment, we adoptively transferred a cytolytically active, MUC1-specific CTL clone into MMT mice [6, 7]. The CTL clone was developed from a MUC1-expressing mouse model that develops spontaneous pancreas tumors (MET mice) [6]. This CTL clone recognizes only an H-2D^b MHC class I-restricted epitope of MUC1 (APGSTAPPA) and expresses Vβ5 and Vα2 [7]. The CTL clone, when adoptively transferred into tumor-bearing MMT mice, homed to the tumor site and lymph node by 2 days post infusion and proliferated as determined by carboxyfluorescein succinimidyl ester (CFSE) labeling ([8] see appendix). CTLs were detected in tumor and lymph node up to 20 days post infusion (Fig. 2). MUC1-specific CTLs (1×10^7) were adoptively transferred into MMT mice at weeks 3, 6, 9, 12, and 15. Mice were sacrificed at 17 weeks and tumor infiltrating lymphocytes (TILs) were isolated and sorted for the infused CD8$^+$ CTL clone based on CD8 and Vβ5 expression (Fig. 3(A), 2nd panel). TILs isolated from age-matched MMT mice that were not infused with CTLs were used as control (4th panel). The CTL clone, maintained in vitro, was the positive control (1st panel). In Fig. 3 we show that the sorted Vβ5/CD8$^+$ TILs had significantly reduced proliferation in response to MUC1 presented on irradiated DCs (Fig. 3B) and were unable to kill tumor cells expressing MUC1 in vitro. However, the CTLs that had not encountered tumor cells and were maintained in tissue culture remained highly cytolytic (Fig. 3C), suggesting that the infused CTLs became non-functional in the tumor environment. The sorted cells also expressed lower levels of granzyme B compared to CTLs maintained in vitro (Fig. 3D).
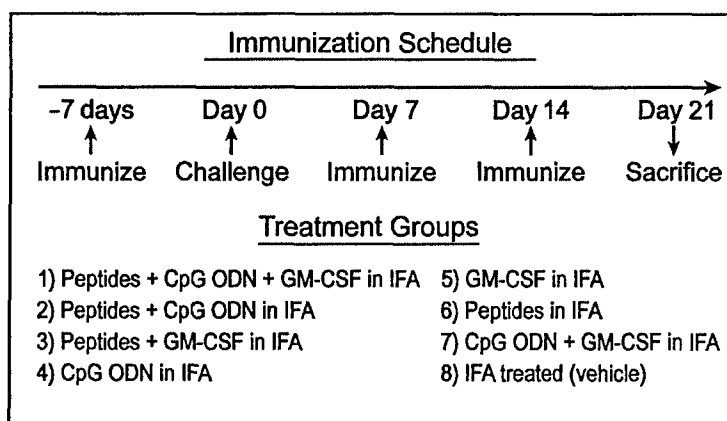
Adoptively Transferred MUC1-specific CTLs Delay Tumor Growth in MMT Mice

There was a significant reduction in tumor growth when CTLs were continuously infused in MMT mice starting at 3 weeks of age (p<0.01, Fig. 4), suggesting that active MUC1-specific CTL were effective in reducing tumor burden and enhancing survival. However, tumors grew out once the CTL infusions were stopped. The resumption of tumor growth without repeated CTL infusion is similar to what was observed in a recent human clinical trial [9]. It is likely that immunotherapy could work if the tumor microenvironment was altered. One way to do this is by co-stimulation for CTL function to overcome the tolerizing effects of the tumor microenvironment.

Reversal of Tolerance

CD137 glycoprotein, a member of the tumor necrosis factor receptor superfamily, is expressed on primed but not on naïve CD4$^+$ and CD8$^+$ T cells. CD137 binds to CD137L expressed on several antigen presenting cells (APCs); activation of CD137 either by ligand binding or by a specific agonistic antibody delivers a dual mitogenic signal for further T cell activation and proliferation. Administration of CD137 mAb can amplify T cell-mediated immune responses, can eradicate established tumors, and functions to reverse established tolerance [10-12]. As we had established that the CTLs in the tumor environment were tolerized, we determined if CD137 mAb could reverse this tolerance. Mice were immunized with CTLs as described and also received 100µg/ml of CD137 mAb intraperitoneally (ip) every week from 3 weeks until sacrifice at 17 weeks. TILs were sorted for Vβ5/CD8$^+$ T cells (Fig. 3A, 3rd panel). CD137 mAb treatment was very effective in reversing tolerance, such that the CTL clone regained responsiveness to MUC1 antigen and lytic activity was restored as shown by ^{51}Cr release and granzyme expression (Fig. 3B,C,D). This reversal of tolerance translated well to anti-tumor response. Tumor burden was significantly lower in CTL + 137 mAb treated mice (p<0.01) and in CTL treated mice (p<0.05) as compared to untreated and CD137mAb treated mice (Fig. 5). Lung metastasis was not evaluated in these mice, since the 17 week end point was earlier than when metastasis is usually detected.

Peptide vaccination against tumor specific antigens, such as MUC1, has little toxicity and may serve as an effective approach to cancer management in the setting of minimal residual disease (post surgical/medical tumor debulking). The expectation is that it will provide fewer side effects than chemotherapy or radiation and may protect against metastasis or recurrence. Peptide-based vaccines provide several advantages over other forms of anti-tumor immunization: (1) peptides are stable, (2) easily synthesized, (3) free of bacterial and other contaminating substances, (4) devoid of oncogenic potential, and (5) provide ease in monitoring the immune response [13, 14].

Immunization Schedule

$$-7 \text{ days} \quad \text{Day 0} \quad \text{Day 7} \quad \text{Day 14} \quad \text{Day 21}$$

-7 days	Day 0	Day 7	Day 14	Day 21
↑	↑	↑	↑	↓
Immunize	Challenge	Immunize	Immunize	Sacrifice

Treatment Groups

1) Peptides + CpG ODN + GM-CSF in IFA   5) GM-CSF in IFA
2) Peptides + CpG ODN in IFA   6) Peptides in IFA
3) Peptides + GM-CSF in IFA   7) CpG ODN + GM-CSF in IFA
4) CpG ODN in IFA   8) IFA treated (vehicle)

Class I and Class II Peptide Immunization

As proof of principle, we have conducted a pre-clinical trial using MUC1-peptide based vaccine in MUC1.Tg mice challenged subcutaneously with 1.5×10^5 MC38 cancer cells transfected with full-length MUC1 gene (MC38.MUC1)or MC38 cells transfected with vector alone (MC38.neo). The trial was conducted in a prophylactic setting. Specifically the vaccine included: a) two MHC class I-restricted MUC1 peptides, APGSTAPPA and SAPDTRPAP, emulsified in Incomplete Freund's Adjuvant (IFA); b) one MHC class II helper peptide TPPAYRPPNAPIL (Hepatitis B virus core antigen sequence 128-140 [15]); c) mouse unmethylated CpG oligodeoxynucleotide constructs (CpG ODN purchased from Coley Pharmaceuticals); and d) granulocyte macrophage-colony stimulating factor (GM-CSF), all emulsified in IFA.

Dose: 100μg MUC1 peptides, 140μg helper peptide, 10,000 Units (2μg) GM-CSF, 100μg of CpG ODN (all emulsified in IFA, 100μl volume). GM-CSF and CpG ODN are given at the same time as the peptides. All injections were administered subcutaneously.
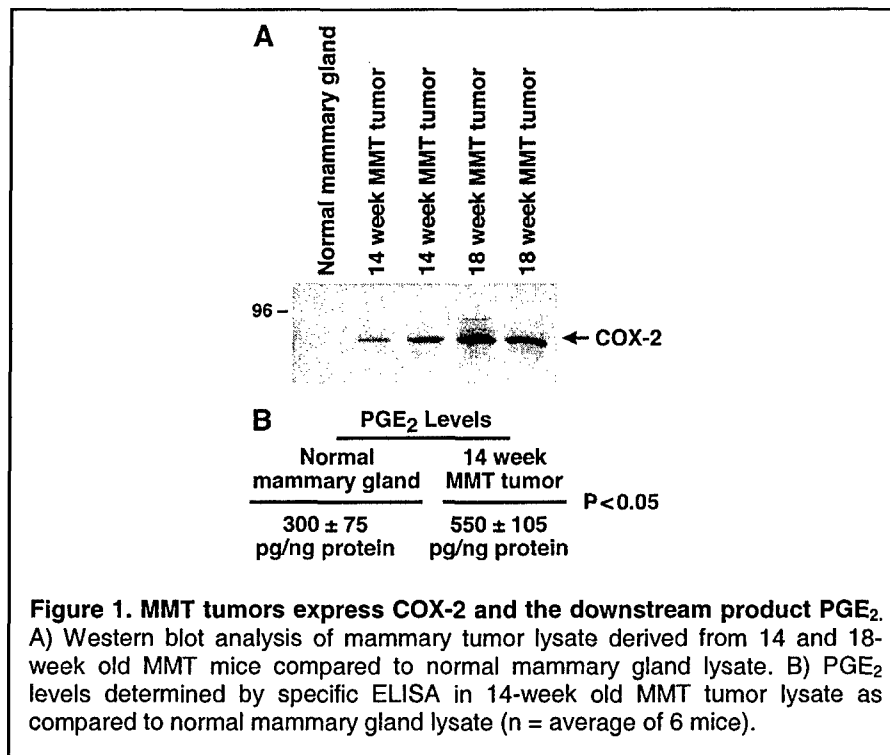
Results demonstrate a complete rejection of MC38.MUC1 tumors in MUC1.Tg mice with MUC1-specific immunization in combination with CpG ODN and GM-CSF ($p<0.001$) (Fig. 6). Mice in all other treatment groups developed tumors although they were significantly different from the vehicle ($p<0.04$) (Fig. 6). To further elucidate the mechanism of anti-tumor response, CD4$^+$ and CD8$^+$ T cells were isolated from lymph nodes of immunized and non-immunized mice and analyzed for a) MUC1-specific IFN-γ spot formation (ELISPOT) and b) lytic potential against specific tumor targets (standard ^{51}Cr release assay). The mechanism of tumor rejection in the MC38.MUC1 challenged mice was determined to be an *in vivo* stimulation of IFN-γ-producing CD4$^+$ helper T cells and CD8$^+$ cytotoxic T cells (Fig. 7A and B). An irrelevant peptide, Vesicular stomatitis virus peptide (RGYKYQGL, VSV NP$_{52-59}$) showed a number of spots similar to vehicle (data not shown).
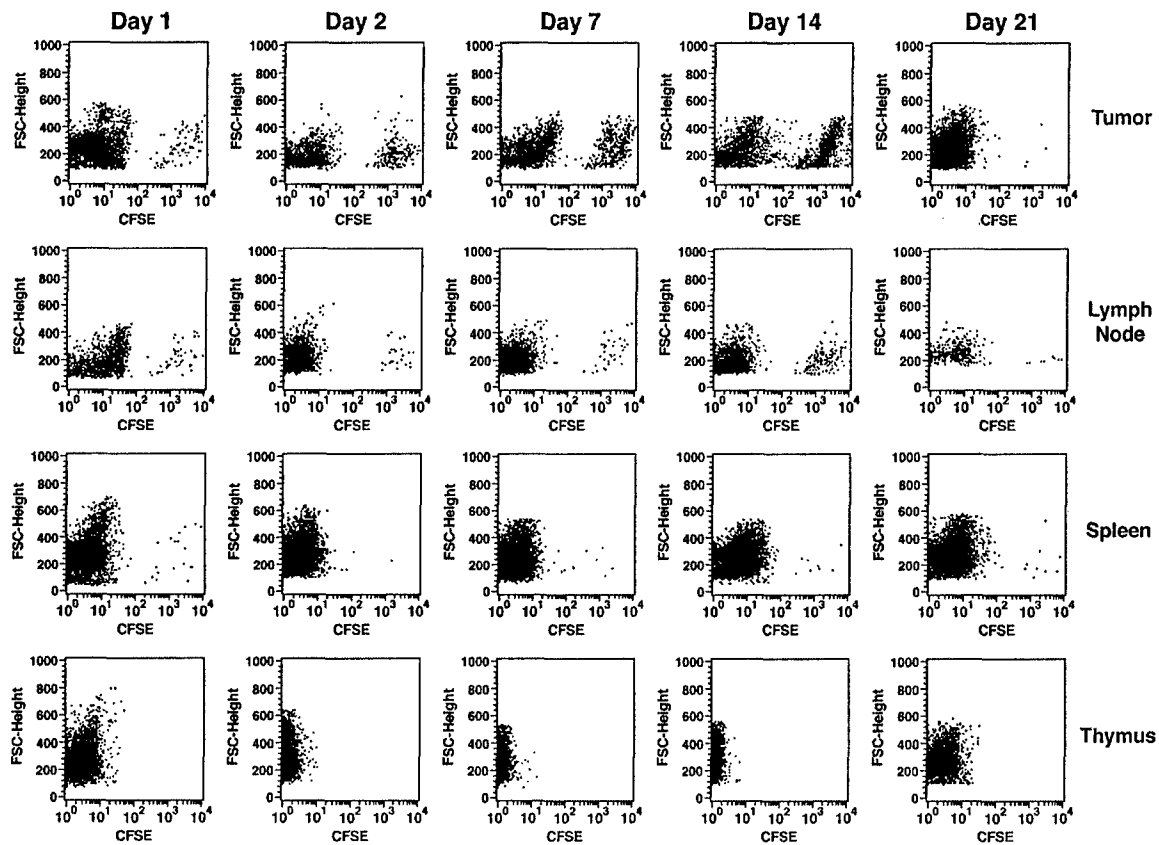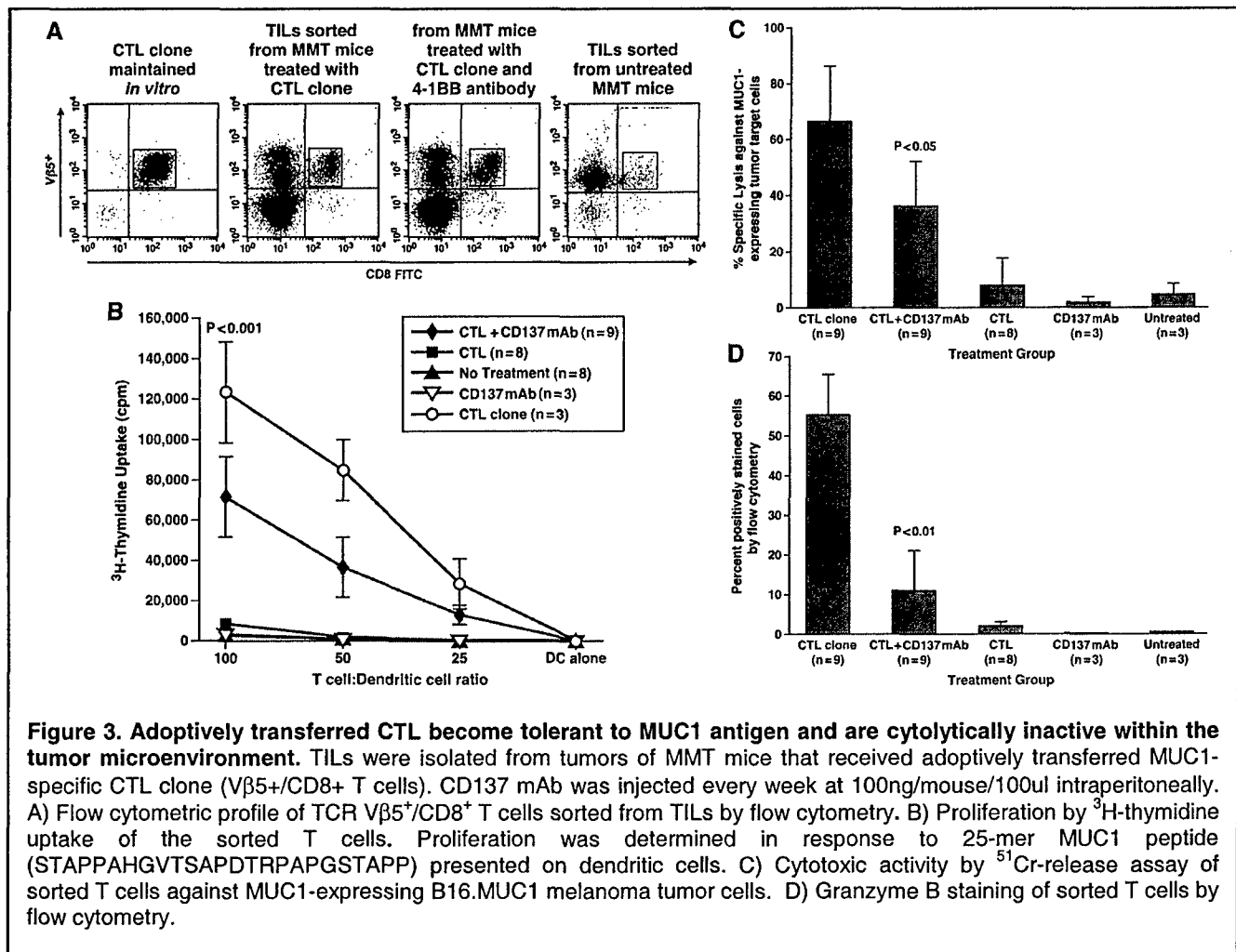
The number of spot forming cells was significantly smaller in the CD8$^+$ T cells than that observed with CD4$^+$ T cells (Fig. 7A and B), suggestive of a strong CD4$^+$ helper T cell response against the pan-helper-peptide and CpG ODN. CpG ODN is also known to target the CD4$^+$ helper T cells, which activate and maintain CD8$^+$ cytotoxic T cells [16-18]. Indeed, when T cells isolated from spleens of immunized mice (both CD4$^+$ and CD8$^+$ T cells) were analyzed for their lytic potential *in vitro*, we observed high MUC1-specific lytic activity directed against MC38.MUC1 tumor cells (Fig. 8). Even more important is that the immunization strategy generated a strong memory response against the MC38.MUC1 tumor cells, such that the mice that were tumor free after primary immunization remained tumor free for months after re-challenge with the same tumor cells (data not shown). Interestingly, the immunization strategy was also successful in significantly reducing MC38.neo tumor growth in MUC1.Tg mice (Fig. 9), presumably due to an immune response elicited by the pan-helper-peptide immunization, CpG ODN and GM-CSF. In the MC38.neo challenged mice, the immunization strongly stimulated the CD4$^+$ helper T cells to produce IFN-γ

(Fig. 10). CD8^{+} cytotoxic T cells did not show as large an increase in IFN-γ spot formation (note different y-axes), suggesting that the immunization was specific for MUC1, and the CD4^{+} T cell response was against the pan-helper peptide. We further determined that the reduced tumor growth in immunized MC38.neo challenged mice was due to the development of lytic T cells (isolated from splenocytes) that specifically lysed MC38.neo target cells in the ^{51}Cr-release assay (Fig. 11). This suggests that the pan-helper peptide-specific immunity is strong in these mice and can translate to an effective anti-tumor response. These results provide strong justification for utilizing an immunization strategy that includes specific class I-restricted CTL epitopes from MUC1 protein, in conjunction with pan-helper peptide, CpG ODN, and GM-CSF in other models, especially in the spontaneous breast cancer model as proposed in this study. This strategy is also similar to that of our current clinical trial (see Aim 4 below). Both CpG ODN and GM-CSF have FDA approval for use in clinical trials.

Figure 1. MMT tumors express COX-2 and the downstream product PGE$_2$. A) Western blot analysis of mammary tumor lysate derived from 14 and 18-week old MMT mice compared to normal mammary gland lysate. B) PGE$_2$ levels determined by specific ELISA in 14-week old MMT tumor lysate as compared to normal mammary gland lysate (n = average of 6 mice).
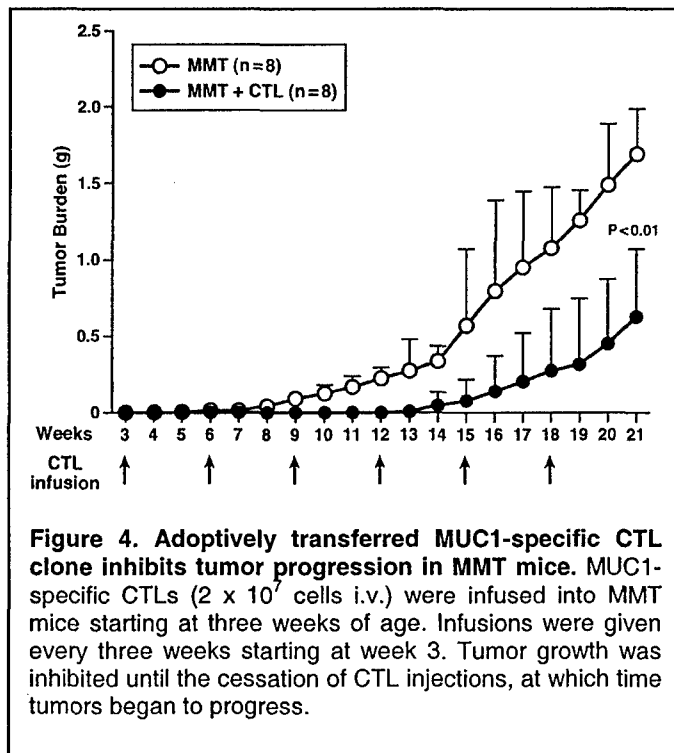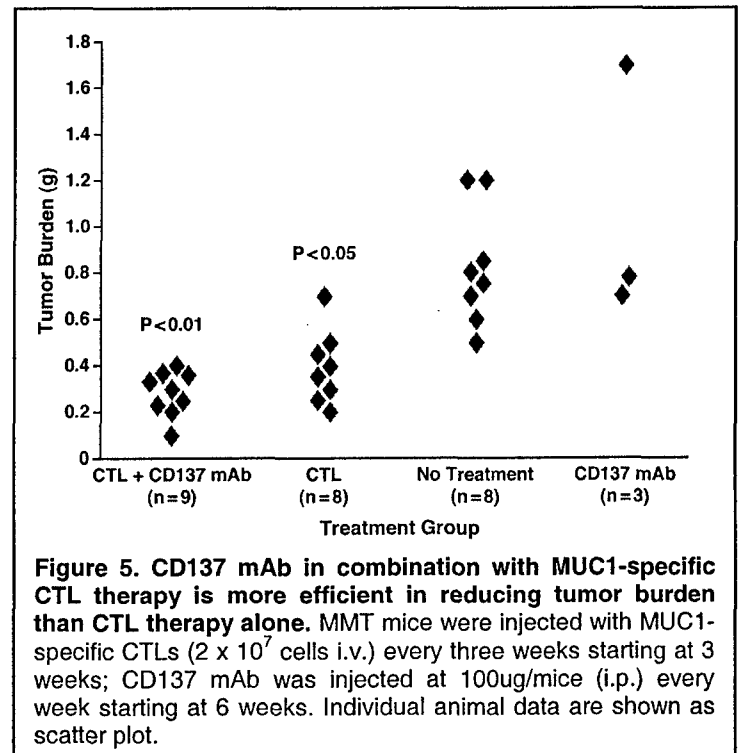
Figure 2. CFSE labeled MUC1-specific CTL home to the lymph node and mammary gland tumor. CTL clone were stained with carboxyfluorescein diacetate succinimidyl ester (CFSE). $2X10^7$ CFSE-stained CTLs were injected intravenously into 14-week old tumor bearing MMT mice. At various times post CTL infusion, TILs, lymph node cells, spleen cells and thymic cells were isolated and monitored for CFSE staining by flow cytometry. n = 5 mice and a representative dot plot is shown.
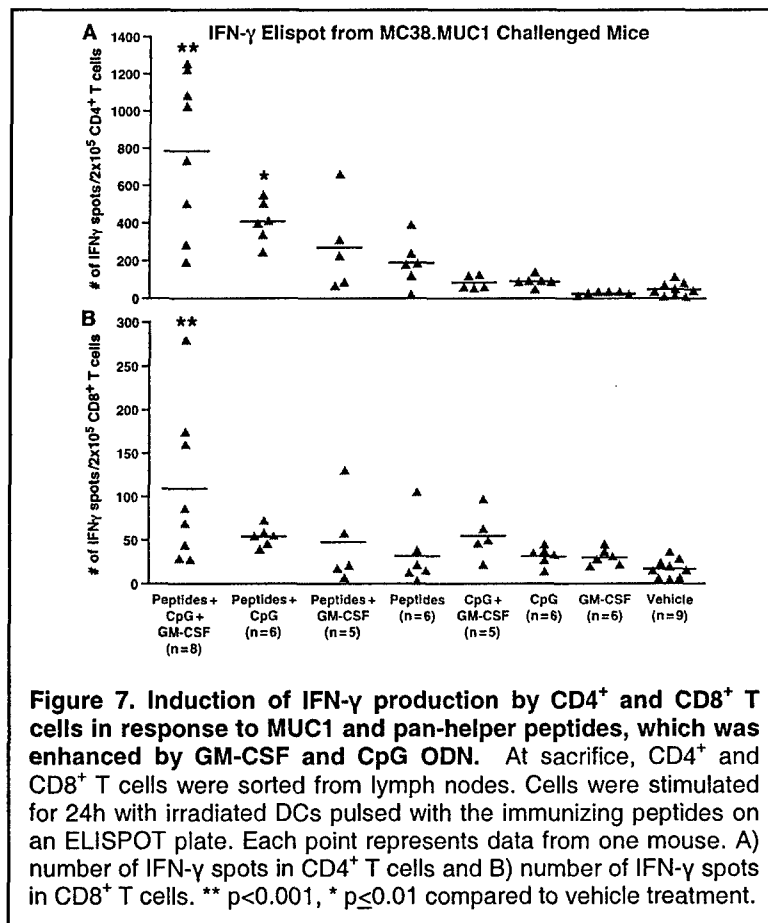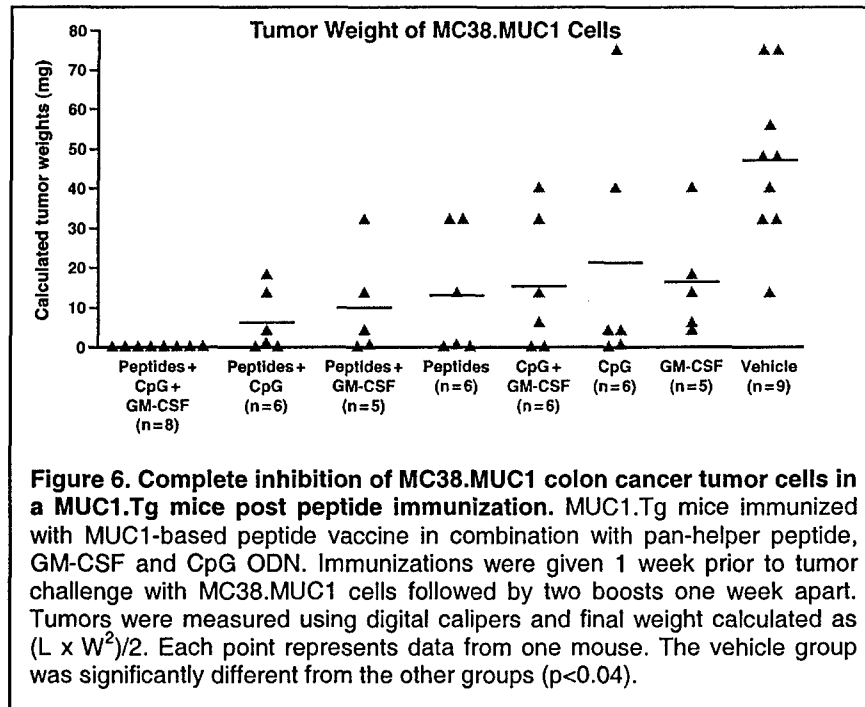
Figure 3. Adoptively transferred CTL become tolerant to MUC1 antigen and are cytolytically inactive within the tumor microenvironment. TILs were isolated from tumors of MMT mice that received adoptively transferred MUC1-specific CTL clone (Vβ5+/CD8+ T cells). CD137 mAb was injected every week at 100ng/mouse/100ul intraperitoneally. A) Flow cytometric profile of TCR Vβ5$^+$/CD8$^+$ T cells sorted from TILs by flow cytometry. B) Proliferation by ^{3}H-thymidine uptake of the sorted T cells. Proliferation was determined in response to 25-mer MUC1 peptide (STAPPAHGVTSAPDTRPAPGSTAPP) presented on dendritic cells. C) Cytotoxic activity by ^{51}Cr-release assay of sorted T cells against MUC1-expressing B16.MUC1 melanoma tumor cells. D) Granzyme B staining of sorted T cells by flow cytometry.
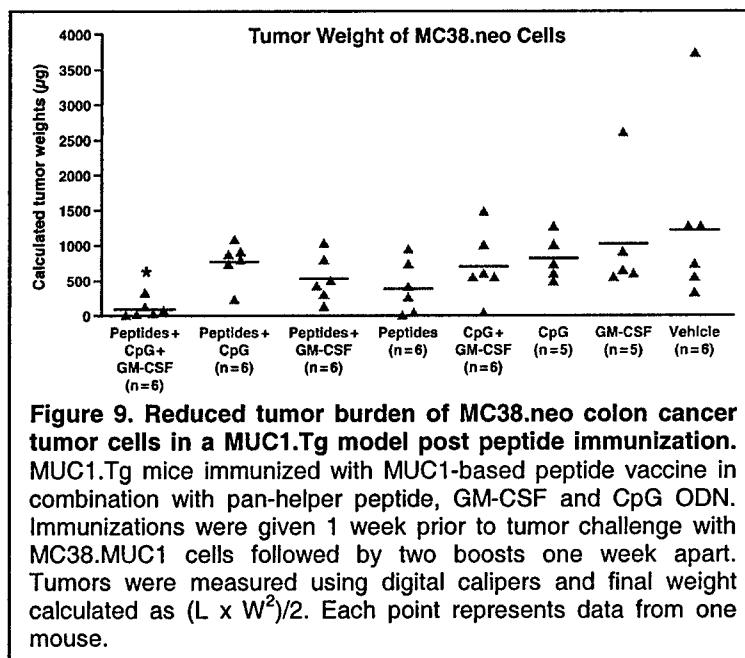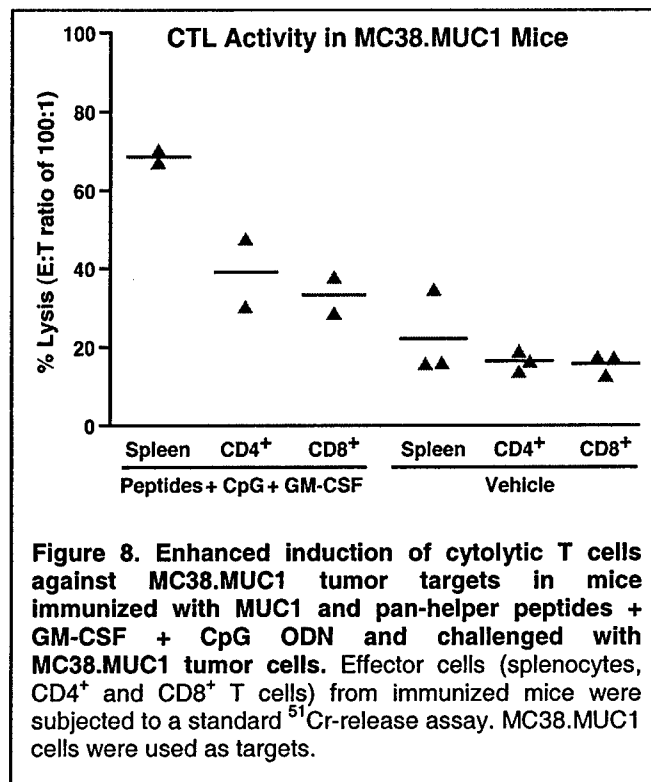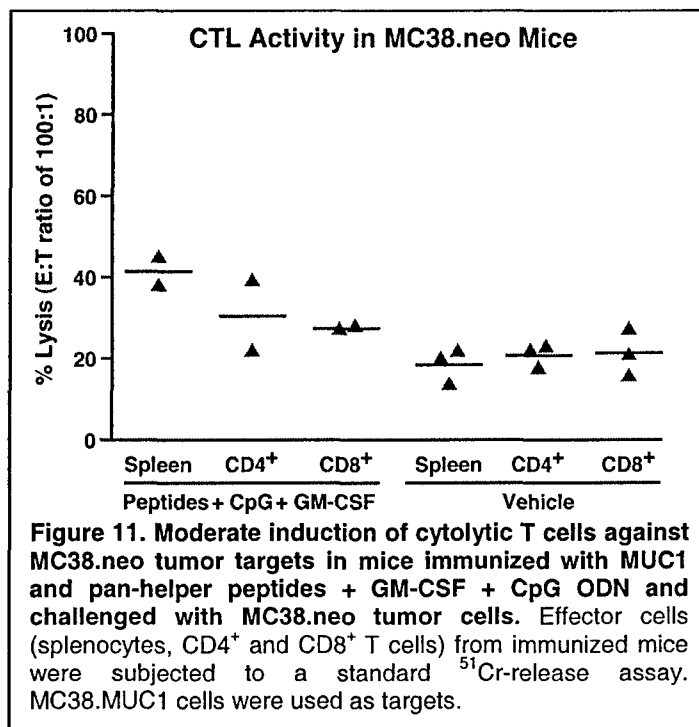

Figure 4. Adoptively transferred MUC1-specific CTL clone inhibits tumor progression in MMT mice. MUC1-specific CTLs (2 x 10^7 cells i.v.) were infused into MMT mice starting at three weeks of age. Infusions were given every three weeks starting at week 3. Tumor growth was inhibited until the cessation of CTL injections, at which time tumors began to progress.

Figure 5. CD137 mAb in combination with MUC1-specific CTL therapy is more efficient in reducing tumor burden than CTL therapy alone. MMT mice were injected with MUC1-specific CTLs (2 x 10^7 cells i.v.) every three weeks starting at 3 weeks; CD137 mAb was injected at 100ug/mice (i.p.) every week starting at 6 weeks. Individual animal data are shown as scatter plot.

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Figure 6. Complete inhibition of MC38.MUC1 colon cancer tumor cells in a MUC1.Tg mice post peptide immunization. MUC1.Tg mice immunized with MUC1-based peptide vaccine in combination with pan-helper peptide, GM-CSF and CpG ODN. Immunizations were given 1 week prior to tumor challenge with MC38.MUC1 cells followed by two boosts one week apart. Tumors were measured using digital calipers and final weight calculated as $(L \times W^2)/2$. Each point represents data from one mouse. The vehicle group was significantly different from the other groups (p<0.04).

Figure 7. Induction of IFN-γ production by CD4$^+$ and CD8$^+$ T cells in response to MUC1 and pan-helper peptides, which was enhanced by GM-CSF and CpG ODN. At sacrifice, CD4$^+$ and CD8$^+$ T cells were sorted from lymph nodes. Cells were stimulated for 24h with irradiated DCs pulsed with the immunizing peptides on an ELISPOT plate. Each point represents data from one mouse. A) number of IFN-γ spots in CD4$^+$ T cells and B) number of IFN-γ spots in CD8$^+$ T cells. ** p<0.001, * p≤0.01 compared to vehicle treatment.

Figure 8. Enhanced induction of cytolytic T cells against MC38.MUC1 tumor targets in mice immunized with MUC1 and pan-helper peptides + GM-CSF + CpG ODN and challenged with MC38.MUC1 tumor cells. Effector cells (splenocytes, CD4$^+$ and CD8$^+$ T cells) from immunized mice were subjected to a standard ^{51}Cr-release assay. MC38.MUC1 cells were used as targets.

Figure 9. Reduced tumor burden of MC38.neo colon cancer tumor cells in a MUC1.Tg model post peptide immunization. MUC1.Tg mice immunized with MUC1-based peptide vaccine in combination with pan-helper peptide, GM-CSF and CpG ODN. Immunizations were given 1 week prior to tumor challenge with MC38.MUC1 cells followed by two boosts one week apart. Tumors were measured using digital calipers and final weight calculated as (L x W^2)/2. Each point represents data from one mouse.

Figure 10. Induction of IFN-γ production by CD4⁺ but not CD8⁺ T cells in response to MUC1 and pan-helper peptides, which was enhanced by GM-CSF and CpG ODN. At sacrifice, CD4⁺ and CD8⁺ T cells were sorted from lymph nodes. Cells were stimulated for 24h with irradiated DCs pulsed with the immunizing peptides on an ELISPOT plate. Each point represents data from one mouse. A) number of IFN-γ spots in CD4⁺ T cells and B) number of IFN-γ spots in CD8⁺ T cells.
** p<0.001, * p≤0.01 compared to vehicle treatment.

Figure 11. Moderate induction of cytolytic T cells against MC38.neo tumor targets in mice immunized with MUC1 and pan-helper peptides + GM-CSF + CpG ODN and challenged with MC38.neo tumor cells. Effector cells (splenocytes, CD4⁺ and CD8⁺ T cells) from immunized mice were subjected to a standard ⁵¹Cr-release assay. MC38.MUC1 cells were used as targets.

Specific Aim 2: To translate the most effective vaccine strategies into phase I clinical trials in patients with high and low tumor burden.

Investigation of immune competence of breast cancer patients In preparation for the clinical trial, Specific Aim 2, we investigated the immune competence of breast cancer patients. This work was described in the report submitted in 2003. The paper describing this work has been published in the Annals of Surgical Oncology. A reprint is supplied in the appendix. The results determined the study population that we will enroll.

Description of Clinical Trial We are including the draft of the clinical trial that is being prepared for submission to the Institutional Review Board (IRB) and to the Federal Drug Administration (FDA) for an IND, which will be pursued concomitantly (see Clinical Trial draft in appendix). Although we originally proposed to use patients with high and low tumor burden, our findings regarding patients with high tumor burden suggested that this category of patient was not optimal for immunotherapy. Patients with breast cancer have T cells that showed decreased proliferation in response to T cell receptor stimulation, dendritic cells with reduced levels of co-stimulatory molecules and reduced ability to mature in response to stimulation, as well as increased PGE_2 (an immunosuppressive protein) levels in circulation. These factors suggest that individuals with tumor burden would not be optimally responsive to immunotherapeutic strategies. Thus, we have chosen to test our cancer vaccine in patients free of detectable breast cancer at the time of registration. Patients will have had histologically confirmed adenocarcinoma of the breast treated with surgery, adjuvant chemotherapy, and/or radiation therapy and have completed "standard therapy" twelve months prior to enrollment in our clinical trial. Patients must have MUC1-positive breast cancer (90% of breast cancers are MUC1 positive) and be HLA-A2 positive. We will enroll 45 patients total at Mayo Clinic Rochester, Mayo Clinic Scottsdale, and Mayo Clinic Jacksonville. We can easily meet this criterion of patient number (see below patient population). Several clinical trials using MUC1 tandem repeat sequences have elicited anti-MUC1 CTLs with limited effects on clinical response. The lack of a clinical response may be due to the patient population being tested (patients with large tumor burden that are immune suppressed) [14].

Patient Population
Breast cancer is the most common type of tumor seen at Mayo Clinic. Considering all three sites, a total of 1,736 new breast cancer patients were seen in 2003 including 1,084 at the Rochester campus, 228 at the Jacksonville campus and 424 at the Scottsdale campus. We have access to all the patients with breast cancer seen at all three Mayo campuses. In addition to a large clinical practice, the Mayo Clinic records system allows review of patient data going back almost one century. We also have data from breast cancer patients entered on prospective clinical trials over the last 30 years. Since 2000 we have prospectively recruited 877 women to a breast cancer patient registry in which a lifestyle and family history questionnaire is obtained. Of these 877 patients, 804 (92%) have provided a blood sample for DNA extraction and 364 patients (42%) have paraffin-embedded tissue available. We have compiled a list of patients that would be eligible for this clinical trial. Once we have completed the review process, these patients will be contacted regarding their interest in participating in the trial. Interest is very high, as immunotherapy has been shown to be a non-toxic therapy.

The trial will test MUC1 class I peptide (STAPPVHNV), HER-2/neu class I peptide (ILHNGAYSL) and HER-2/neu class II peptide (KVPIKWMALESIL) (1000 µg or each peptide) delivered in Montanide ISA-51 and compare GM-CSF with unmethylated CpG oligodeoxynucleotides as immune adjuvants. Few vaccines have been tested in the optimal setting of minimal residual disease. We feel this is a strength of this trial. The clinical trial entitled "MUC1/HER-2/neu Peptide Based Immunotherapeutic Vaccines for Breast Adenocarcinomas" is included in the appendix. It is still in draft form as we are awaiting the quality control information for the MUC1 peptide prior to submission to the FDA for an IND. It will be submitted to the Mayo IRB concurrently with the FDA submission. We will then submit to the Human Subject Research Review Board. We were substantially delayed by the failure of two manufacturing companies to synthesize

the MUC1 signal sequence peptide, LLLLTVLTV, to sufficient purity. Thus, we changed to the above listed MUC1 peptide, which is a degenerate tandem repeat peptide.

The schema for the clinical trial is shown (Fig. 12).

Figure 12
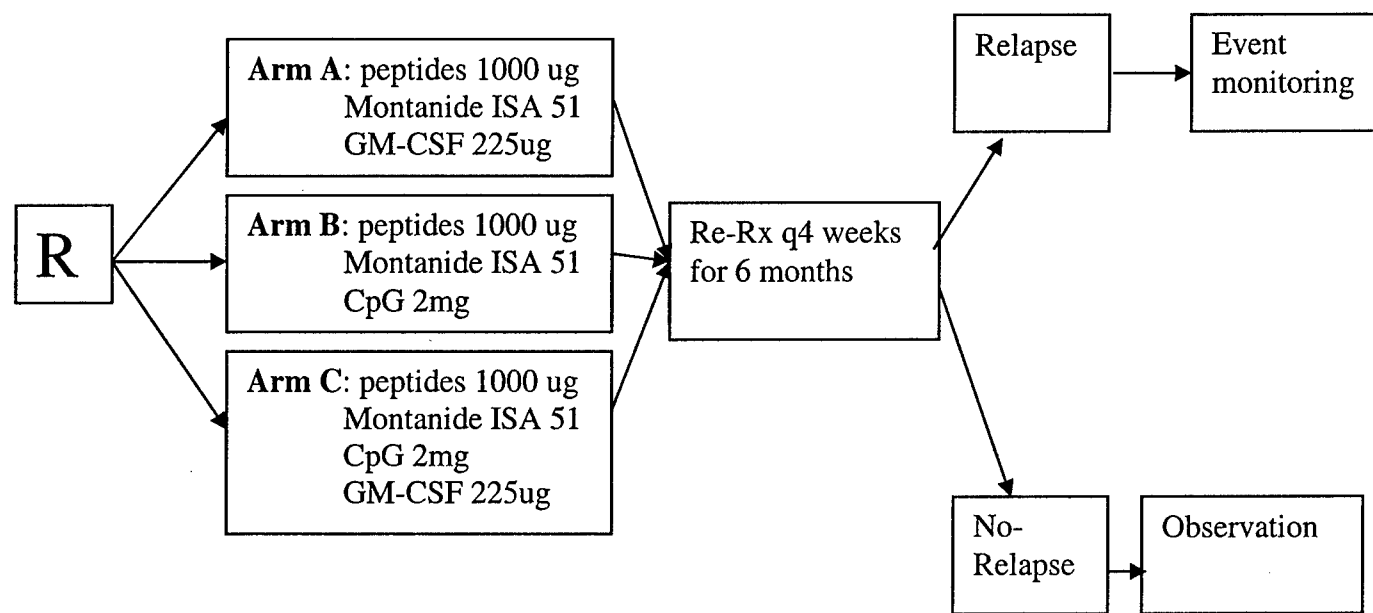
KEY RESEARCH ACCOMPLISHMENTS

- Two additional immunization strategies have been tested in our mouse model of spontaneous mammary gland cancer (MMT mice).

    o Adoptive transfer of MUC1-specific cytotoxic T lymphocytes
    o Immunization with MHC class I- and class II-binding (MUC1) peptides, CpG ODN and GM-CSF

- Tumor microenvironment induced tolerance as shown by failure of T cells to proliferate to MUC1 and reduced lytic capabilities.

- Cross-linking of co-stimulatory molecules strategies to reverse established tolerance were tested.

- Adoptive transfer of MUC1-specific CTL clone inhibited tumor progression in MMT mice

- CD137 antibody plus MUC1-specific CTLs were effective in:

    o Significantly reducing tumor burden in immunized MMT mice
    o Overcoming T cell anergy/tolerance

- MUC1 peptide immunization elicited complete rejection of MUC1-expressing tumor cells injected into MUC1.Tg mice

- MUC1 peptide immunization stimulated a strong immune response as evidenced by

    o In vivo stimulation of IFN-γ-producing CD4$^+$ helper T cells and CD8$^+$ cytotoxic T cells
    o Increased lytic activity

- Adjuvants CpG ODN and GM-CSF enhanced the immune response in the peptide vaccine trial

- Investigation of T cell and DC functionality in breast cancer patients at time of diagnosis compared to age-matched volunteers showed reduced functionality.

- A draft of the Clinical Trial is included in the appendix

Time Table of Protocol Development

Completed

- Clinical protocol concept approved by Mayo Clinic Cancer Center 12-11-03

- Completed Mayo Clinic Cancer Center Peer Review process   5-4-04

- List of recommendations by FDA (pre IND conference)   4-21-04

- Peptides synthesized and vialed by ClinAlfa® for use in this clinical trial:
    1. Her-2/neu (435-443)   7-16-04
    2. Her-2/neu (883-899)   8-6-04
    3. MUC1(950-958)   PENDING

<u>Pending</u> (upon completion of MUC1 peptide synthesis and issue of "Certificate of Analysis")

- Completion of IND documentation and submission to FDA

- Upon FDA approval, protocol submission to DOD and IRB

- Upon approval by DOD and IRB, study activation

REPORTABLE OUTCOMES

Pockaj, B.A., Basu, G.D., Pathangey, L.B., Gray, R.J., Hernandez, J.L., Gendler, S.J. and Mukherjee, P. (2003) Reduced T cell and dendritic cell function is related to COX-2 over-expression and PGE2 secretion in patients with breast cancer. Annals of Surgical Oncology 11:328-339

Mukherjee, P., Tinder, T.L., Basu, G.D., Pathangey, L.B., Chen, L. and Gendler, S.J. (2004) Terapeutic efficacy of MUC1-specific cytotoxic T lymphocytes and CD137 co-stimulation in a spontaneous breast cancer model. Breast Disease, In Press.

Basu, G.D., Pathangey, L.B., Tinder, T.L., LaGioia, M., Gendler, S.J., and Mukherjee, P. COX-2 inhibitor induces apoptosis in breast cancer cells in an in vivo model of spontaneous metastatic breast cancer. Molecular Cancer Research, In Press.

Mukherjee, P., Tinder, T.L., Basu, G.D., Pathangey, L.B., Chen, L., and Gendler, S.J. Therapeutic efficacy of MUC1-specific cytotoxic T lymphocytes and CD137 co-stimulation in a spontaneous mammary cancer model. International Society for Biological Therapy of Cancer, abstract accepted for platform presentation, Nov. 2004.

CONCLUSIONS

All of the vaccine strategies have elicited an immune response. Animals developed mature CTLs which were lytic *in vitro* against MUC1-expressing tumor cells. Lytic activity was detected without further *in vitro* stimulation. However, in most cases the spontaneous tumors progressed. The CTLs, while active outside of the environment of the tumor, were tolerized and unreactive to MUC1 (a target antigen) in the vicinity of the tumor. Tumors were found to exhibit several known escape mechanisms, such as the production of immunosuppressive factors such as Cox-2 and PGE_2 and down-modulation of MHC class I molecules on the cells. CTLs that were adoptively transferred into the tumor-bearing mice were used to follow the development of tolerance, which occurred within about three weeks following injection. Stimulation of co-stimulatory molecules, especially CD137, a member of the TNFR family, together with adoptive transfer of MUC1-specific T cells, resulted in significantly reduced tumor burden in MMT mice. The CD137-stimulated CTLs appeared to remain lytic against the tumor in the tumor environment and tolerance/anergy was alleviated. CTLs (tumor infiltrating lymphocytes) recovered from the tumors were lytic against MUC1-expressing tumor cells (MMT tumor cells in culture, as well as B16/MUC1 cells) and they proliferated in response to DCs presenting MUC1. A peptide vaccine was tested in MUC1.Tg mice using MUC1-expressing tumors injected subcutaneously. Two class I-binding peptides from MUC1 (APGSTAPPA and SAPDTRPA from the MUC1 tandem repeat region) and a class II helper peptide (TPPAYRPPNAPIL) were used together with CpG ODN and GM-CSF in Incomplete Freund's Adjuvant. The peptide vaccine completely inhibited the growth of MUC1-expressing tumor cells and elicited a strong immune response. This strategy will be utilized in the clinical trial described in Aim 2).

We showed reduced functionality of T cells and dendritic cells in breast cancer patients with large tumor burden. Ours was the first study to evaluate DC and T cell function from the same breast cancer patients and from age-matched controls. We chose to limit the study population to patients with resected stage II or III breast cancer who completed "standard therapy" 12 months previously and have no evidence of disease. This time lapse should allow the immune system to recover to normal levels prior to the first immunization. The draft clinical trial is included in the appendix. GMP-grade peptide synthesis has been completed for all three peptides. We are waiting for the "Certificate of Analysis" for the MUC1 peptide prior to submission to the FDA for an IND. Submission to the Mayo IRB will be made simultaneously, followed by submission to the DOD Human Subjects Research Review Board. We anticipate that the trial will open in early 2005. We presently have a list of patients who would be eligible for this trial. We anticipate that there will be rapid accrual to this trial.

Future Studies

The clinical trial will open shortly. We will enroll patients free of detectable breast cancer at the time of registration. Patients will have had histologically confirmed adenocarcinoma of the breast treated with surgery, adjuvant chemotherapy, and/or radiation therapy and have completed "standard therapy" twelve months prior to enrollment in our clinical trial. Patients must have MUC1-positive breast cancer (90% of breast cancers are MUC1 positive) and be HLA-A2 positive (45% of people express the A2 allele). We will enroll 45 patients total at Mayo Clinic Rochester, Mayo Clinic Scottsdale, and Mayo Clinic Jacksonville. We can easily meet this criterion of patient number. The trial will test MUC1 class I peptide, HER-2/neu class I peptide and HER-2/neu class II peptide (1000 µg) delivered in Montanide ISA-51 and compare GM-CSF, CpG ODN or CpG ODN together with GM-CSF as immune adjuvants. Few vaccines have been tested in the optimal setting of minimal residual disease. We feel this is a strength of this trial. The draft of the clinical trial entitled "MUC1/HER-2neu Peptide Based Immunotherapeutic Vaccines for Breast Adenocarcinomas" is included in the appendix.

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APPENDICES

Pockaj, B.A., Basu, G.D., Pathangey, L.B., Gray, R.J., Hernandez, J.L., Gendler, S.J. and Mukherjee, P. (2004) Reduced T cell function is related to COX-2 over-expression and PGE_2 secretion in patients with breast cancer. Ann Surg Oncol 11:328-339.

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Draft of Clinical Trial – MUC1/HER-2/neu Peptide Based Immunotherapeutic Vaccines for Breast Adenocarcinomas

Draft – Patient Consent Form

Letter to Myung-Joo Patricia Hong with "Responses to pre-IND meeting summary letter of 4-21-04"

Certificate of Analysis for HER 2/neu (435-443)

Certificate of Analysis for HER 2/neu (883-899)

Copy of Peptide Vial Labels

Letter from Coley Pharmaceutical Group, Inc. regarding cross-reference for CPG 7909

Reduced T-Cell and Dendritic Cell Function Is Related to Cyclooxygenase-2 Overexpression and Prostaglandin E$_2$ Secretion in Patients With Breast Cancer

Barbara A. Pockaj, MD, Gargi D. Basu, PhD, Latha B. Pathangey, MS, Richard J. Gray, MD,
Jose L. Hernandez, BA, Sandra J. Gendler, PhD, and Pinku Mukherjee, PhD

Background: In several neoplastic diseases, including breast cancer, immunosuppression correlates with disease stage, progression, and outcome. Thus, thorough analysis of immune parameters in breast cancer patients may be beneficial in designing effective anticancer immune-based therapies.

Methods: We investigated dendritic cell and T-cell function in breast cancer patients at various stages of the disease and in age-matched controls. We also evaluated cyclooxygenase-2 (COX-2) expression and prostaglandin E2 (PGE$_2$) levels within the tumor milieu and in the circulation.

Results: T cells from cancer patients showed decreased proliferation in response to CD3 antibody stimulation. Analysis of T-cell helper type 1 and 2 cytokines revealed reduced levels of interferon-γ, tumor necrosis factor-α, interleukin (IL)-12, and IL-2 and increased levels of IL-10 and IL-4. Dendritic cells from these patients showed significantly reduced expression of co-stimulatory molecules (B7 and CD40) and demonstrated reduced phagocytic ability, reduced antigen presentation to T cells, and reduced ability to mature in response to lipopolysaccharide. Data revealed increased synthesis of PGE$_2$, an immune suppressor, along with increased expression of COX-2, a key regulator of PGE$_2$ synthesis.

Conclusions: COX-2–induced PGE$_2$ may contribute to immunosuppression and may directly block antitumor immunity while promoting tumor growth, providing us with the rationale for using COX-2 inhibition combined with immunotherapy.

Key Words: Cyclooxygenase-2—Prostaglandin E$_2$—Dendritic cells—T cells—Breast cancer.

The defective function of the host's immune system is one of the major mechanisms by which tumors evade immune surveillance. T-cell anergy is thought to be an early event in tumor progression and may precede the generalized immunosuppression that is observed in cancer patients.[1] Multiple mechanisms of tumor-specific evasion have been demonstrated, including impaired HLA expression, modulation of surface antigens, lack of co-stimulatory molecules on dendritic cells (DCs) leading to impaired antigen presentation to T cells, impaired T-cell receptor (TCR) signal transduction through the TCR-ζ chain, and elaboration of immune-suppressive cytokines by tumor cells and T-regulatory cells such as interleukin-10 (IL-10) and transforming growth factor-β (TGF-β).[2]

Functional impairment of T cells has been well documented in patients with cancer.[3] This is true of both circulating and infiltrating lymphocytes in which there is reduced activation of the TCR and impaired production of interferon-γ (IFN-γ), IL-2, and tumor necrosis factor-α (TNF-α) in response to CD3 monoclonal antibodies.[4] Cytokine profiles of cancer patients demonstrate an abnormal balance between T-cell helper type 1 (Th1) and type 2 (Th2) cytokines, favoring a Th2 response.[5] Circulating and tumor-infiltrating DCs have also been

shown to be functionally impaired. In metastatic melanoma patients, tumor-infiltrating DCs express low levels of co-stimulatory molecules (CD80 and CD86) and therefore are unable to activate T cells, whereas DCs isolated from breast cancer patients demonstrate a significantly reduced ability to stimulate allogeneic and antigen-specific T-cell responses.[6,7] In certain cancers, DCs derived from peripheral blood are lower in absolute number as compared with those in individuals without cancer and are predominantly immature in phenotype.[8]

Cyclooxygenase-2 (COX-2) is overexpressed in a variety of cancers, including breast cancer.[9-11] COX-2 is an enzyme that converts arachidonic acid to prostaglandin H_2, which is further metabolized to other prostaglandins, including prostaglandin E_2 (PGE_2).[12] COX-2 expression is rapidly induced secondary to a number of factors, including growth factors, tumor promoters, and hormones.[13] Transgenic mouse models demonstrated that overexpression of COX-2 leads to the development of mammary tumors.[14] Overexpression of COX-2 is also known to inhibit apoptosis[15] and promote angiogenesis.[16] This overexpression of COX-2 can lead to increased production of prostaglandins such as PGE_2, which has multiple downstream effects. PGE_2 is known to transactivate the epidermal growth factor receptor, which triggers mitogenic signaling in epithelial cells and induces cancer cell proliferation.[17] PGE_2 also causes immunosuppression in vitro[18] and can induce immunosuppression in vivo, enhancing tumor growth in animal models.[19,20] In this study, we tested the hypothesis that the COX-2–induced PGE_2 overexpression may correlate with the global immunosuppression observed in breast cancer patients.

Because T cells and DCs are pivotal in the development of antitumor immunity and are susceptible to tumor-mediated immune suppression, we investigated DC and T-cell function from 25 breast cancer patients at various stages of the disease and compared the data with those of 19 healthy age-matched controls. Although several studies have described the functional impairment of T cells and DCs in breast cancer patients, the studies have not evaluated both T-cell and DC function from the same breast cancer patients. Moreover, the mechanisms driving the functional impairment still remain elusive. The goal of our study was to evaluate the immune status of patients presenting with the diagnosis of breast cancer and to evaluate the immune-modulating factors within the tumor milieu that may account for the functional impairment of immune effector cells. This is the first study to describe a thorough analysis of both T-cell and DC function in patients with newly diagnosed breast cancer. Impaired functionality of T cells and DCs corre-

lated with COX-2 and PGE_2 overexpression. These studies are of critical importance for designing novel immunotherapeutic strategies for breast cancer and for selecting the patients who may most benefit from such therapies.

METHODS

Study Characteristics

This research study was approved by the Mayo Clinic Institutional Review Board. Patients who presented to the Mayo Clinic Scottsdale Breast Clinic for initial treatment of disease were eligible for the protocol. The patients signed informed consent for peripheral blood and tumor samples. Informed consent for peripheral blood samples was also obtained from healthy, age-matched volunteers. Twenty-five patients with breast cancer and 19 healthy controls were studied. Patients and healthy donors who were chronic users of COX-2 inhibitors were excluded from the analysis. Staging of the patients was conducted by using the American Joint Committee on Cancer protocol. Breast cancer patient demographics and tumor characteristics are listed in Table 1. All patients were postmenopausal, with a mean age of 69 years. Controls were all postmenopausal, with a mean age of 60 years. Infiltrating ductal adenocarcinoma was the most common tumor subtype (64%). The mean size of the tumors was 2.3 cm, although 64% of lesions were ≤2 cm. Only 20% of patients had lymph node metastases,

TABLE 1. *Patient demographics and tumor characteristics*

Variable	Data
Age, y, median (range)	69 (36–80)
Presentation	
Mass	12 (48%)
Abnormal mammogram	13 (52%)
Tumor size, cm, median (range)	2.3 (0.3–13)
Tumors ≤2 cm	16 (64%)
Histology	
Infiltrating ductal	16 (64%)
Infiltrating lobular	3 (12%)
Mixed infiltrating ductal/lobular	3 (12%)
Infiltrating mucinous	3 (12%)
Grade	
1	9 (36%)
2	8 (32%)
3	8 (32%)
Angiolymphatic invasion	4 (16%)
Estrogen receptor positive	21 (84%)
Lymph node metastases	5 (20%)
Number of lymph nodes positive	1–8
Stage	
I	15 (60%)
II	7 (28%)
III	3 (12%)

and most patients presented with either stage I or stage II disease. In most cases, blood was drawn on the day of surgery, before resection of the tumor. In some cases, blood was drawn few days before surgery.

The overall scheme for the study design is shown in Fig. 1. Whole blood was obtained from the study subjects, and the peripheral blood mononuclear cells (PB-MCs) were separated by using a Ficoll-Paque density gradient centrifugation. Sera from these patients were also collected and stored in a $-80°C$ freezer. The PB-MCs were used for isolation of T cells and DCs, and the serum was used for cytokine/chemokine and PGE_2 evaluation. Surgically resected tumor tissues and lymph node metastases were used to make tissue lysate for COX-2 and PGE_2 evaluation.

T-Cell Isolation

T cells were isolated from the PBMCs of patients as described in the scheme (Fig. 1). Briefly, mononuclear cells were obtained by centrifugation of peripheral blood over a Ficoll-Paque gradient (Amersham Biosciences, Uppsala, Sweden). Mononuclear cells were incubated for 2 hours at 37°C, and nonadherent lymphocytes were removed and used as T cells and adherent cells were used for generation of DCs. The processing of the blood was performed under the same conditions, and all assays were performed on freshly isolated buffy-coat cells.

T-Cell Proliferation Assay

The nonadherent lymphocyte population (1×10^6/mL) was subjected to in vitro stimulation with various concentrations of purified plate-bound CD3 antibody (BD Pharmingen, San Diego, CA). Cells were incubated for 4 days with CD3 antibody, and ^{3}H-thymidine was added 24 hours before collection. After excess thymidine was washed off, cells were lysed with 5% Triton X-100 (Fischer Scientific, Pittsburgh, PA), and incorporated thymidine was evaluated with the Topcount micro scintillation counter (Packard Biosciences, Shelton, CT). Evaluation of T-cell proliferation was also performed with varying concentrations of tumor lysates (12–200 μg/mL) and purified PGE_2 (Cayman Pharmaceuticals, Ann Arbor, MI). All assays were performed in triplicate. Control lymphocytes were included in every assay to control for interassay variation.
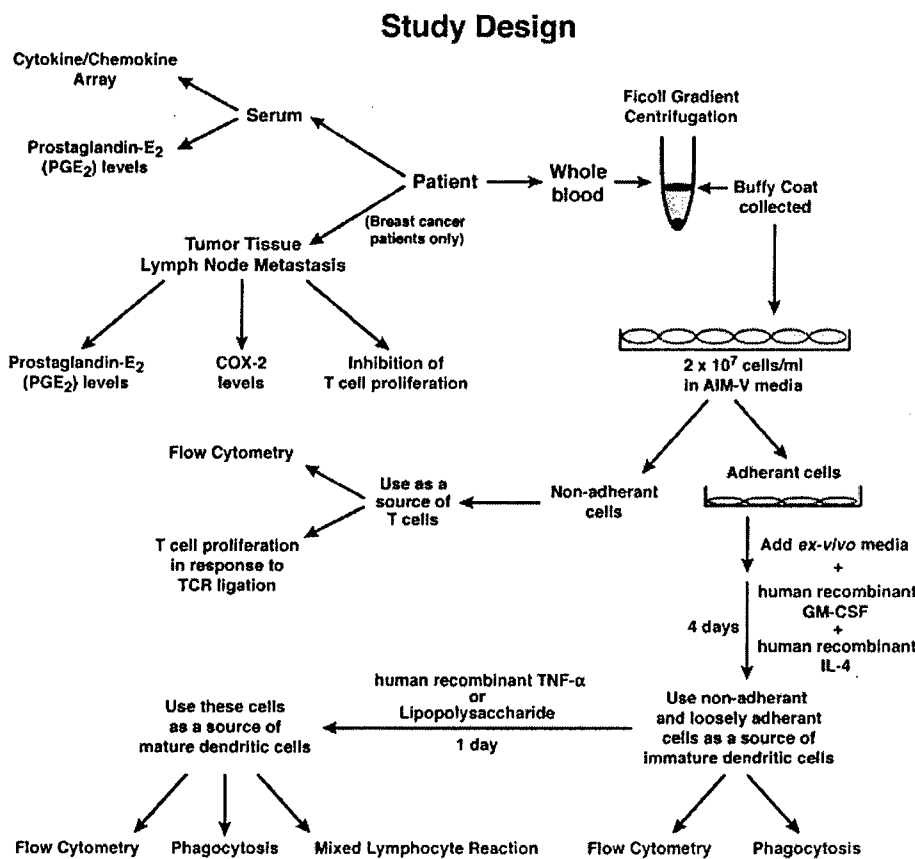
Study Design

FIG. 1. Schematic representation of the study design. COX-2, cyclooxygenase-2; TCR, T-cell receptor; GM-CSF, granulocyte-macrophage colony-stimulating factor; IL, interleukin; TNF, tumor necrosis factor; AIM-V, serum free human lymphocyte culture media.

Analysis of Intracellular Cytokines

Intracellular cytokine levels were evaluated by two-color flow cytometric analysis after TCR ligation. Intracellular cytokines were determined post brefeldin A (BD Pharmingen) treatment of lymphocytes according to the manufacturer's recommendations (4 μL/1.2 $\times$ 10^7 cells per 6 mL for 3 hours at 37°C before staining). This treatment stops the release of cytokines in the culture media, and the cytokines accumulate within the cells. Cells were then stained for surface markers for T cells (CD3) or DCs (HLA-DR) at 4°C for 15 minutes, followed by washing excess stain and permeabilizing cells with Pharmingen permeabilization solution (containing saponin) for 30 minutes at 4°C. Cells were then stained for intracellular IL-2, IL-12, IFN-γ, IL-4, IL-10, and TNF-α for 30 minutes at 4°C. Cells were analyzed with the Becton Dickinson FACScan, and data were analyzed with the CellQuest program. All antibodies were purchased from BD Pharmingen.

Serum Analysis of Cytokines and Chemokines

A cytokine/chemokine array kit (Ray Biotech Inc., Norcross, GA) was used to detect a panel of 22 secreted cytokines and chemokines in the serum from healthy patients and those with breast cancer. The manufacturer's recommended protocol was used.

DC Isolation and Maturation

DCs were generated from a CD14$^+$ monocyte population isolated from PBMCs. Briefly, mononuclear cells were obtained by centrifugation of the peripheral blood over a Ficoll-Paque gradient. Mononuclear cells were incubated for 2 hours at 37°C, and nonadherent cells were removed. Adherent cells were incubated with granulocyte-macrophage colony-stimulating factor (5 ng/mL; PeproTech, Rocky Hill, NJ) and IL-4 (5 ng/mL; PeproTech) for 4 to 5 days. Cells were collected, counted, and phenotyped for immature DCs and were further cultured for one additional day with granulocyte-macrophage colony-stimulating factor (5 ng/mL), IL-4 (5 ng/mL), and lipopolysaccharide (LPS, 100 ng/mL; Sigma Pharmaceuticals, St. Louis, MO). Cells were collected on day 6 as mature DCs.

DC Phenotype

Control and breast cancer patient DCs were analyzed by two-color flow cytometric analysis. Cell-surface expression of several markers was evaluated: CD80 (B7.1), CD86 (B7.2), CD40, HLA-DR, HLA-ABC, CD1a, and CD14. All antibodies were purchased from BD Pharmingen. Stained cells were analyzed with the CellQuest program on a Becton Dickinson FACScan.

DC Function

Mixed Lymphocyte Reaction Assay

Control and patient-derived DCs were assayed for their ability to stimulate allogeneic T cells in a mixed lymphocyte reaction (MLR). T cells (1 $\times$ 10^5) from healthy donors were incubated with irradiated DCs (3000 rads; 1 $\times$ 10^4 cells) from allogeneic breast cancer patients for 5 days, and ^{3}H-thymidine was added 24 hours before the cells were collected. After the excess thymidine was washed off, cells were lysed with 5% Triton X-100, and incorporated thymidine was evaluated by using the Topcount micro scintillation counter.

Phagocytosis Ability

Immature and LPS-matured DCs from healthy donors and breast cancer patients were incubated with fluorescein isothiocyanate–conjugated dextran beads (molecular weight, 40,000; Molecular Probes Inc., Eugene, OR) at 1 mg/1 $\times$ 10^6 cells for 30 minutes at 37°C. Dextran beads were used as the exogenous antigen source. Because the beads were conjugated to fluorescein isothiocyanate, uptake of dextran beads by DCs was analyzed by flow cytometry, and mean fluorescence intensity was calculated.

Breast Tumor Cell Lysates

Tissue lysates were prepared within 1 hour after surgery by homogenizing the tumor tissue in lysate buffer containing 20 mM of 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid, .15 M of NaCl, and 1% Triton X-100 supplemented with phosphatase-inhibitor cocktail mix (1/100 dilution; Sigma Pharmaceuticals) and complete protease inhibitors (Roche Pharmaceuticals, Indianapolis, IN). Lysates were stored in a -80°C freezer for further use.

Expression of COX-2 Protein in Tumor Lysate

Protein concentrations of the lysates were determined with the Pierce BCA protein assay kit (Pierce, Rockford, IL). Sodium dodecyl sulfate-polyacrylamide gel electrophoresis was performed with 12% resolving gel; 100 μg of protein was loaded per lane. Gels were immunoblotted and probed for COX-2 with specific COX-2 monoclonal antibody (goat polyclonal antibody, clone C20; Santa Cruz Biotechnology Inc., Santa Cruz, CA) at a 1/200 dilution.

PGE$_2$ in Serum and Tumor Lysate

Levels in the lysates were determined with a specific enzyme-linked immunosorbent assay (ELISA) kit for PGE$_2$, and levels in serum were determined by using the

PGE$_2$ metabolite ELISA kit (Cayman Pharmaceuticals). The manufacturer's recommended protocols were followed.

Statistical Analysis

Statistical significance was assessed by using pairwise comparisons with the Tukey-Kramer adjustment for multiple comparisons. The margins of error for the comparisons were obtained by calculating the 95% confidence intervals for the differences between group proportions.

Immune function parameters were compared between a set of clinical indicators. The clinical parameters examined were stage, lymph node status, estrogen receptor status, tumor size (<2 vs. 2 cm), grade, presence of angiolymphatic invasion, multifocality, and previous breast cancer. Because of the nonnormality of the immune function data and the small sample size of the cohort, the exact Wilcoxon statistic was used for assessing significant differences between groups. All error bars in the figures represent the standard deviation of the mean.

RESULTS

The proliferative ability of T cells isolated from breast cancer patients just before surgical tumor resection was examined. T cells were stimulated by various concentrations of plate-bound CD3 antibody, and T-cell proliferation was measured by ^{3}H-thymidine uptake. T-cell proliferation was significantly reduced in cancer patients compared with controls ($P < .001$ at 1 µg and .5 µg/mL of CD3 antibody; Fig. 2A). The raw counts per minute for all patients (n = 25) and controls (n = 19) are presented in Fig. 2B for a 1 µg/mL CD3 antibody concentration. Figure 2B clarifies the number of patients who were immunosuppressed. Sixty percent (16 of 25) of the breast cancer patients had lower than 50,000 counts per minute, which is suggestive of impaired T-cell proliferation in response to TCR ligation. All controls had values greater than this value. Additional analysis of Th1/Th2 cytokines in activated cells revealed reduced intracellular levels of the immunostimulatory Th1 cytokines IFN-γ ($P < .001$), TNF-α ($P < .001$), IL-12 ($P < .001$), and IL-2 ($P < .001$) and increased levels of the Th2 cytokines IL-4 ($P = .1$) and IL-10 ($P = .01$; Fig. 3).

DCs from patients with breast cancer expressed reduced levels of co-stimulatory molecules such as CD80 (B7.1), CD86 (B7.2), and CD40 on maturation with LPS (Fig. 4). Similar results were obtained with TNF-α–induced maturation. Other markers used to determine DC phenotype included HLA-DR, HLA-ABC, CD1a, and CD14 (data not shown). No differences between cancer
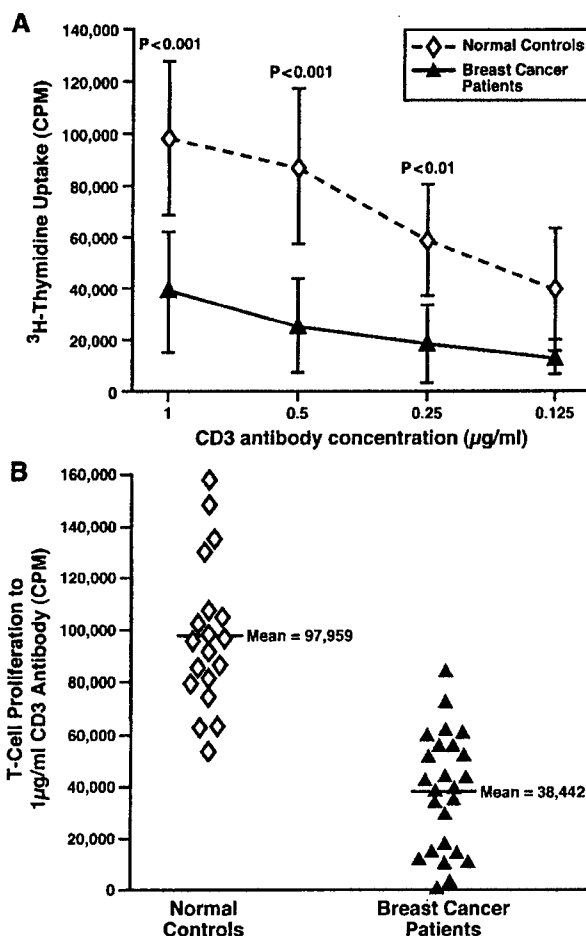

FIG. 2. T cells from breast cancer patients have markedly reduced proliferation in response to specific T-cell receptor ligation. (A) T-cell proliferation in response to plate-bound CD3 antibody comparing breast cancer patients (n = 25) and controls (n = 19). The amount of ^{3}H-thymidine uptake directly corresponds to the proliferative capacity of T cells. Error bars represent standard deviation of the mean. All assays were performed in triplicate with interassay controls. (B) Scatter plot of ^{3}H-thymidine uptake (in counts per minute; CPM) of T cells from individual patients (n = 25) and healthy donors (n = 19) in response to 1 µg/mL of plate-bound CD3 antibody.

patients and controls were observed in these markers. The reductions in expression of both CD80 and CD86 were statistically significant ($P < .001$). This low expression of co-stimulatory molecules is an indication that these DCs remain immature. It has been suggested (1) that immature DCs have a reduced ability for stimulating T cells and therefore may contribute to tumor-induced T-cell tolerance rather than immunity and (2) that mature DCs are essential for presenting tumor antigens and activating T cells to become cytolytic against tumor cells.

We therefore tested whether DCs from cancer patients had reduced antigen presentation. Data indicate that the
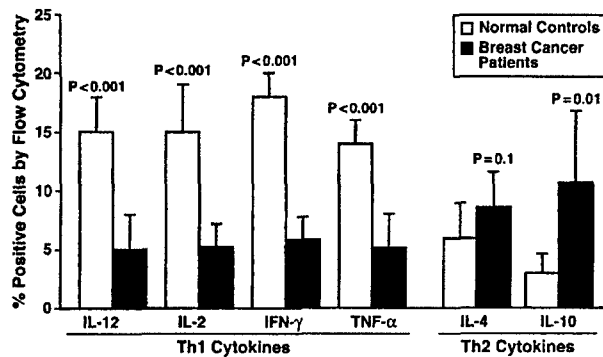
FIG. 3. Reduced T-helper type 1 (Th1) cytokines and increased T-helper type 2 (Th2) cytokines in the peripheral blood of patients with breast cancer. Intracellular cytokine levels were compared by flow cytometry. Interferon (IFN)-γ, tumor necrosis factor (TNF)-α, interleukin (IL)-12, IL-2, IL-10, and IL-4 levels were compared between breast cancer patients (solid bars; n = 9) and controls (open bars; n = 7). Error bars represent standard deviation of the mean.

function of DCs was significantly reduced. DCs from breast cancer patients demonstrated a significantly reduced ability to present antigens to allogeneic normal T cells in an MLR ($P < .001$; Fig. 5A). The MLR results support the previous findings shown in Fig. 4, in which the same DCs expressed low levels of co-stimulatory molecules and therefore failed to fully mature. Furthermore, the immature DCs from breast cancer patients demonstrated a significantly reduced ability to phagocytose exogenous antigens in vitro ($P < .001$; Fig. 5B and
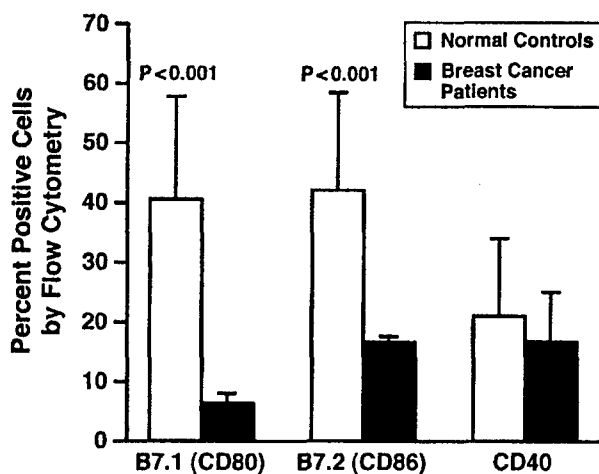
FIG. 4. Dendritic cells (DCs) from patients with breast cancer expressed reduced levels of co-stimulatory molecules. Flow cytometric analysis is shown of the surface expression of the co-stimulatory molecules CD80 (B7.1), CD86 (B7.2), and CD40 on lipopolysaccharide-matured DCs of breast cancer patients (solid bars; n = 25) versus controls (open bars; n = 19). Similar results were obtained with tumor necrosis factor-α–matured DCs (data not shown). Error bars represent standard deviation of the mean.
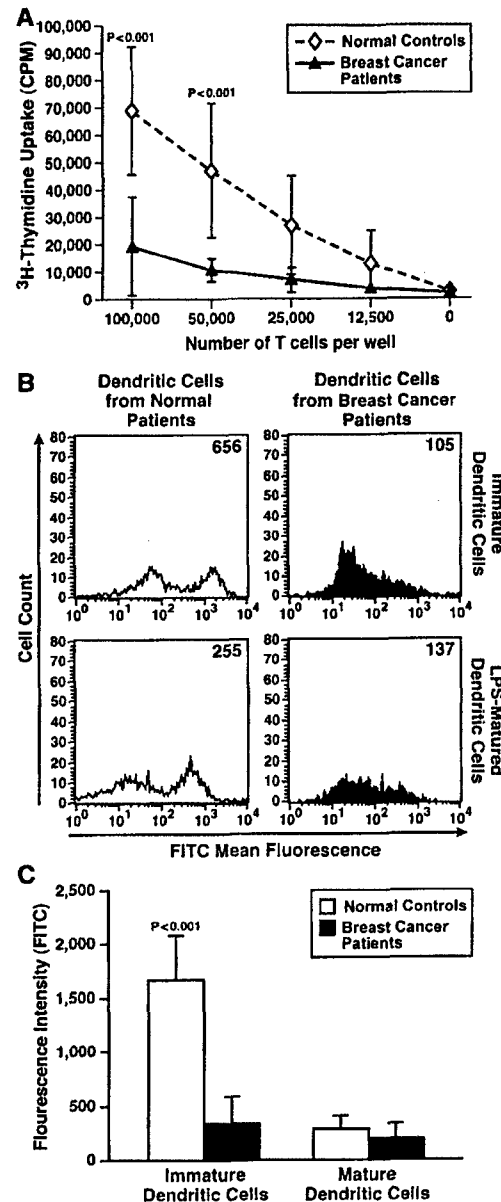

FIG. 5. Dendritic cells (DCs) from breast cancer patients demonstrate a significantly reduced ability to present antigens to allogeneic normal T cells and demonstrate reduced phagocytosis of exogenous antigen. (A) Allogeneic antigen presentation to normal T cells by DCs of breast cancer patients (n = 25) versus controls (n = 19) in a mixed lymphocyte reaction. The amount of ^{3}H-thymidine uptake directly corresponded to the proliferative capacity of T cells. (B) Representative histogram of immature and lipopolysaccharide-matured dendritic cell phagocytic ability of a cancer patient versus healthy donor (the numbers on the right corner are the mean fluorescence intensity). (C) Dendritic cell phagocytic ability (mean fluorescence intensity) in breast cancer patients (solid bars; n = 25) versus normal controls (open bars; n = 19) in the mature and immature state. In (B) and (C), the mean fluorescence intensity was used as a measure for the amount of fluorescein isothiocyanate–conjugated dextran beads engulfed by the DCs. Error bars represent standard deviation of the mean. CPM, counts per minute; FITC, fluorescein isothiocyanate.

C). They also demonstrated a maturation defect when stimulated with LPS treatment (Fig. 5C). Figure 5B shows a representative histogram from 1 patient, and Fig. 5C shows a bar graph of average values from the 24 patients and 19 controls. Immature DCs are known to be strong phagocytes but weak antigen presenters, whereas mature DCs are weak phagocytes and strong antigen presenters.[21] We found that immature DCs from healthy donors showed significantly higher ($P < .001$) fluorescence intensity (mean intensity of 1436), thus demonstrating good phagocytic ability, and on maturation with LPS, their phagocytic activity was reduced (mean intensity of 291; Fig. 5C). In comparison, the immature DCs from the breast cancer patients had significantly lower fluorescence intensity (mean intensity of 337), thus indicating poor phagocytosis; the florescence intensity of these DCs did not decrease with LPS treatment and remained at mean intensity of 207, once again indicating impaired maturation (Fig. 5C). Similar results were obtained when TNF-α instead of LPS was used to mature DCs (data not shown).

Tumor cells secrete factors that are known to induce immunosuppression and promote tumor cell proliferation. Prostaglandins, especially PGE$_2$, are such factors expressed within the tumor microenvironment and secreted in the serum. Because COX-2 is the key regulator of prostaglandin synthesis, we evaluated the COX-2 protein expression on Western blots of adjacent normal tissue lysates, tumor tissue lysates, and lysates from lymph nodes that contained metastases. The COX-2 protein was overexpressed in both the tumor and lymph node metastases compared with normal tissue. The highest expression was observed in lymph node metastases (Fig. 6A). Next, we evaluated the amount of PGE$_2$ in the serum of breast cancer patients and healthy donors by specific PGE$_2$ metabolite ELISA. Because PGE$_2$ is rapidly converted in the serum by 15-OH prostaglandin dehydrogenase to its 13,14-dihydro-15-keto metabolite, direct measurement of intact PGE$_2$ is not possible in sera or plasma. Thus, PGE$_2$ metabolite measurement is necessary to provide a reliable estimate of actual PGE$_2$ production.[22,23] However, in the tumor tissue lysates, PGE$_2$ levels can be evaluated directly by using a specific ELISA for PGE$_2$, because these cell types do not contain the enzymes required for metabolism of PGE$_2$, thus keeping the PGE$_2$ levels stable. Breast cancer patients had significantly increased levels of PGE$_2$ metabolite levels in their sera as compared with controls ($P < .001$; Fig. 6C). Similar to COX-2 expression, we observed high levels of PGE$_2$ in the tumor cell lysates ($P = .042$) and lymph node metastases ($P = .017$) as compared with normal adjacent breast tissue (Fig. 6B).
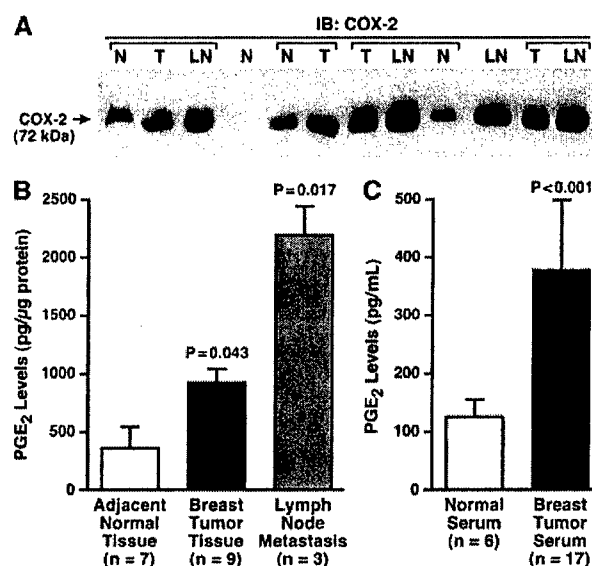
FIG. 6.   Cyclooxygenase-2 (COX-2) and prostaglandin E$_2$ (PGE$_2$) are overexpressed in the tumor tissue and serum of patients with breast cancer. (A) COX-2 protein levels in tissue lysates (100 μg) from adjacent normal breast tissue, breast tumors, and lymph node metastases were analyzed by Western blot analysis. Brackets indicate tissues from a single patient. Six patient samples were analyzed. (B) PGE$_2$ levels in tissue lysates from adjacent normal breast tissue, breast tumors, and lymph node metastases were determined by specific enzyme-linked immunosorbent assay (ELISA). (C) Serum PGE$_2$ metabolite levels in breast cancer patients (solid bars) versus controls (open bars) were analyzed by specific PGE$_2$ metabolite ELISA. Error bars represent standard deviation of the mean. N, normal adjacent tissue; T, tumor; LN, lymph node metastasis; IB, immunonoblot.

Because tumor lysates contained COX-2, PGE$_2$, and, presumably, other immunosuppressive factors, we determined whether these lysates could directly block the T-cell signal transduction and activation that lead to T-cell proliferation. We evaluated the effect of the tumor lysates on the proliferation of T cells from the healthy donors. We used purified PGE$_2$ as a standard and compared it with the inhibition observed with the tumor lysate and lysate derived from adjacent normal tissue. There was direct inhibition of normal T-cell proliferation in response to CD3 antibody by the tumor lysates (n = 3) and lysates derived from lymph node metastasis (n = 2) at a 25 μg/mL concentration (Fig. 7). As expected, inhibition was also observed with purified PGE$_2$ at the same concentration (Fig. 7). Little inhibition of T-cell proliferation was observed with lysates derived from adjacent normal tissue, which did not express high levels of PGE$_2$ by ELISA (Fig. 6B). The inhibition observed with tumor and lymph node metastasis lysate was higher than with purified PGE$_2$, suggesting the presence of other T cell–inhibitory agents in the tumor lysate. Because normal lymph nodes express some PGE$_2$, normal
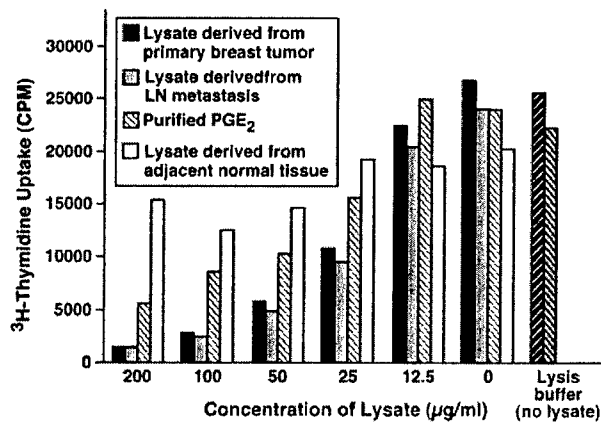
FIG. 7. Direct inhibition of normal T-cell proliferation by factors present in the tumor lysate. T-cell proliferation was determined in response to plate-bound CD3 antibody in the presence or absence of lysate derived from the primary tumor, lysate derived from lymph node (LN) metastasis, lysate derived from adjacent normal tissue, or purified prostaglandin E_2 (PGE$_2$) at varying concentrations (0 –200 µg/mL). T cells were generated from healthy donors (n = 4). The amount of ^{3}H-thymidine uptake directly corresponds to the proliferative capacity of T cells. Tissue lysis buffer and lysate from adjacent normal tissue were used as negative controls, and purified PGE$_2$ was used as the positive control. This assay was repeated three times, with similar results. CPM, counts per minute.

adjacent tissue may not be the ideal control for lymph node metastasis lysate. Thus, we evaluated normal lymph node lysates from mice (because normal human lymph nodes were not available) and determined that the PGE$_2$ levels in normal lymph nodes were minimal and were not sufficient to cause inhibition of mouse T-cell proliferation in response to TCR ligation (data not shown). Moreover, in humans, it has been well established that normal T cells that have not undergone any antigenic challenge or environmental insult generate very little PGE$_2$. In vitro studies have proven that normal T cells have to be stimulated with mitogen to express PGE$_2$.[24,25] Thus, we are confident that the inhibition of T-cell proliferation that we observed with lysates from lymph node metastasis was due to high levels of PGE$_2$ (Fig. 6B) and other unknown immunosuppressive factors.

We evaluated our data to see whether the patient's clinical variables correlated with immune function parameters. The clinical parameters examined were stage, lymph node status, estrogen receptor status, tumor size (≤2 vs. 2 cm), grade, presence of angiolymphatic invasion, multifocality, and previous breast cancer. Immune function variables were compared between a set of clinical indicators. Because of the nonnormality of the immune function data and the small sample size of the cohort, the exact Wilcoxon statistic was used in assessing

significant differences between groups. However, no significant correlation was reached except for impaired DC phagocytosis and antigen presentation in patients with lymph node–positive disease versus those who were lymph node negative ($P = .025$). The inability to elucidate any differences in any other parameters and clinical factors is due to small sample size.

DISCUSSION

There is evidence that tumor-specific antigens are present on cancer cells that could function as potential targets for the immune system. Unfortunately, cancer patients do not mount an effective immune response against them, indicating that the immune cells are tolerant to the tumor-specific antigens. Breaking this tolerance is one of the major goals of immunotherapy for cancer. Tumors also exhibit multiple immunosuppressive strategies, such as downregulation of major histocompatibility complex class I molecules, lack of co-stimulatory molecules on DCs, and secretion of immunosuppressive cytokines, as well as production of high levels of COX-2 and PGE$_2$. We first examined the immune status of patients recently diagnosed with breast cancer and then evaluated the effect of COX-2 overexpression by the tumor cells and subsequent synthesis of PGE$_2$ on the tumor's ability to evade immune surveillance.

Overall we found significant functional impairment in the T cells of patients who were diagnosed with breast cancer. T cells isolated from breast cancer patients before surgical removal of the tumor demonstrated a marked reduction in their proliferation response to CD3 antibodies (Fig. 2), suggesting a defect in activation of the TCR-mediated signal transduction pathways.[26] These described defects include reduced TCR-ζ chain expression, a defect in transcription factors such as nuclear factor-κB, upregulation of cyclin-dependent kinase inhibitor p27kip1, and hydrogen peroxide production by activated granulocytes.[26–30] A likely consequence of this ineffective T-cell signaling is impaired cytokine production by the T cells.[5,31] Nieland et al.[31] found that reduced cytokine expression was found in patients with early breast cancer with a normal TCR-ζ chain, suggesting involvement of other mechanisms in causing impaired cytokine production.

Th1 cytokines promote the development of cell-mediated antitumor responses.[32] However, Th2 cytokines are necessary for humoral immunity. Patients with carcinoma have a predominance of Th2 cytokines in the peripheral blood.[5] We found a similar increase in Th2 cytokines (IL-10 and IL-4) in our breast cancer cohort when compared with controls and found reduced Th1

cytokines (IFN-γ, IL-2, and IL-12; Fig. 3). A shift to a Th2 response has been correlated with increasing stage in patients with renal cell carcinoma.[33] Preliminary analysis of serum chemokine and cytokine levels by using a cytokine array system revealed a correlation between increasing levels of certain cytokines and chemokines with advanced-stage breast cancer (Fig. 8). RANTES (regulated on activation, normal T cells expressed and secreted), monokine induced by IFN-γ, monocyte chemoattractant protein-1, IL-8, and IL-10 levels (proteins that favor a Th2 response) were higher in the sera from patients with high-grade tumor and lymph node metastases as compared with patients with low-grade tumor and no lymph node metastases. Monocyte chemoattrac-

tant protein-1 is implicated in tumor cell migration and invasion and in multidrug resistance.[34] Similarly, monokine induced by IFN-γ and RANTES favor inflammation and tumor cell proliferation and invasion.[35,36] IL-8 is implicated in increased angiogenesis and multidrug resistance,[34] and IL-10 is known to cause T-cell anergy.[37] TGF-β1 is known to downregulate both cytotoxic T lymphocyte and T-helper signal transduction and function.[38]

These chemokines and cytokines are released by monocytes, macrophages, and lymphocytes that express the EP receptors for binding to PGE₂.[39] It is therefore plausible that the high levels of PGE₂ observed within the tumor microenvironment and in the circulation may
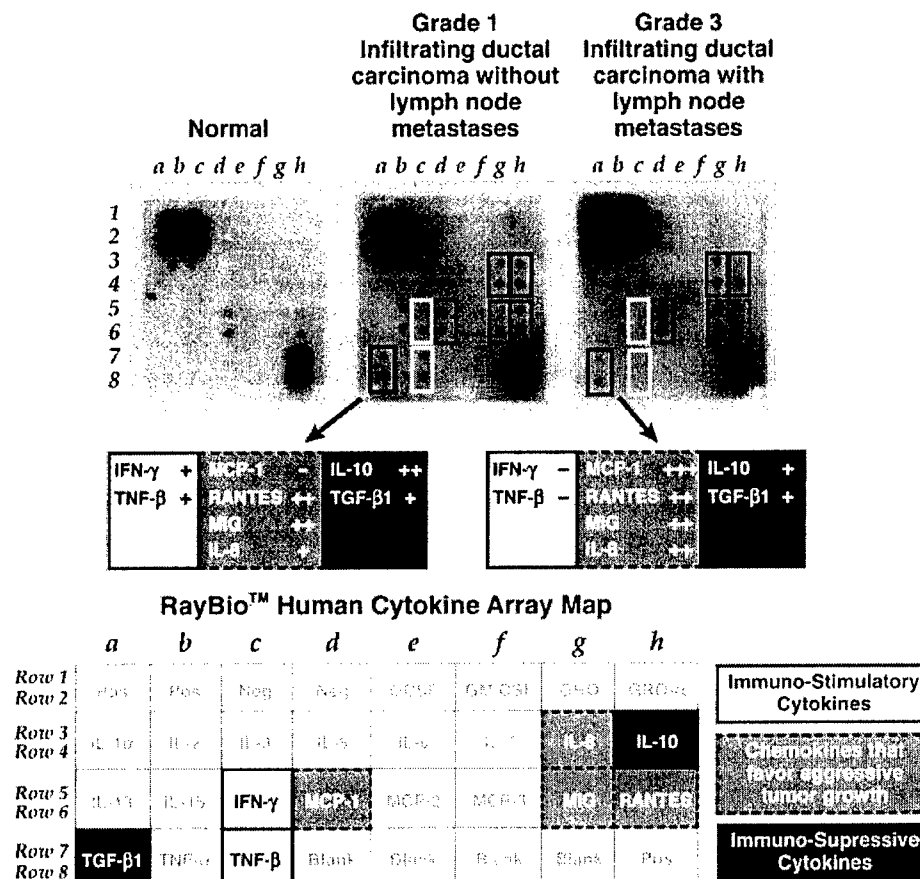
FIG. 8. Serum analysis of the chemokine/cytokine array revealed a correlation between increasing levels of certain cytokines and chemokines and advanced-stage breast cancer. Expression of a panel of 22 secreted cytokines and chemokines was detected in the serum of healthy and breast cancer patients by using the Ray Biotec cytokine array kit. Sera are shown from one control, one patient with a grade 1 invasive breast cancer without lymph node metastases, and one patient with a grade 3 invasive breast cancer with lymph node metastases. Similar results were observed with the other five breast cancer patients tested. The boxes on the blots and table demonstrate the cytokines and chemokines that are either upregulated (+) or downregulated (−) compared with the normal serum. The open box represents the immunostimulatory cytokines, the gray box represents chemokines that favor aggressive tumor growth, and the black box represents immunosuppressive cytokines. The actual cytokine array map from Ray Biotec is also provided. IFN, interferon; TNF, tumor necrosis factor; MCP, monocyte chemoattractant protein; RANTES, regulated on activation, normal T cells expressed and secreted; MIG, monokine induced by interferon-γ; IL, interleukin; TGF, transforming growth factor; GCSF, granulocyte colony-stimulating factor; GM-CSF, granulocyte-macrophage colony-stimulating factor; GRO, growth-related oncogene.

activate the tumor-infiltrating lymphocytes, monocytes, and macrophages via the EP receptors to release factors that favor tumor growth and invasion and suppress immune effector cells. These proteins were either absent or present in very low levels in the sera of controls. Conversely, serum levels of the immunostimulatory cytokines TNF-β1 and IFN-γ were higher in patients with low-grade/nonmetastatic tumors when compared with patients with high-grade/metastatic tumors, once again suggesting that advanced-stage patients are more likely to be immunosuppressed. The cytokine/chemokine array analysis was performed on six breast cancer patients and three healthy donors. Representative blots from two cancer patients and one healthy donor are shown in Fig. 8. The tumor milieu has been thought to play a significant role in the impairment of DCs. Release of such factors as IL-6, IL-10, macrophage colony-stimulating factor, vascular endothelial growth factor, and macrophage inflammatory protein-3α can prevent DC maturation and antigen-presenting functions. It is interesting to note that in our preliminary analysis of sera from four patients with breast cancer, we observed increased levels of vascular endothelial growth factor, macrophage colony-stimulating factor, IL-10, and macrophage inflammatory protein-3α (data not shown) as compared with controls. These results fit well with the concept that these increased chemokine levels favor a Th2 response that limits the DC allostimulatory capability.[7,40–42] In one study, it was shown that surgical removal of the primary tumor resulted in a dramatic reduction in the proportion of immature DCs, although the levels never reached those of controls.[8]

Mature DCs are the most powerful antigen-presenting cells and thus initiate the immune response.[21] The presence of immature DCs is thought to contribute to the induction of tolerance instead of immunity against the tumor antigens.[43] Low expression of co-stimulatory molecules on circulating DCs is an indication of immaturity. Low levels of co-stimulatory molecules in peripheral and draining lymph node DCs of breast cancer patients and impaired allostimulatory ability have been demonstrated in patients with breast cancer.[7,8] Our study confirms some of these findings as shown by the low expression of co-stimulatory molecules on circulating DCs from our breast cancer patients (Fig. 4). Our data further demonstrate the immaturity of DCs by the fact that DCs from cancer patients have a reduced ability to present antigens to allogeneic normal T cells in an MLR assay (Fig. 5A).

Heightened endocytic activity is characteristic of cytokine-derived DCs and their enhanced capacity to capture and process antigens.[21] Our study demonstrated impaired phagocytosis by the immature DCs from breast cancer patients (Fig. 5B and C). Attempts to mature the DCs with LPS did not change their phagocytic ability, once again suggesting a defect in DC maturation.

The mechanisms that underlie the T-cell and DC anergy in cancer patients are unknown but probably involve multiple events. We evaluated whether overexpression of COX-2 and downstream PGE$_2$ synthesis may be one of the mechanisms for immunosuppression. It is interesting to note that the COX-2 expression was high in primary tumors and was even more prominent in lymph node metastases (Fig. 6A). Because COX-2 was overexpressed, we evaluated the PGE$_2$ levels in the serum of the breast cancer patients and controls along with tumor lysates. Increased levels of PGE$_2$ were demonstrated in both the sera and tumor lysates of patients with cancer (Fig. 6B and C). PGE$_2$ is an immune suppressor that targets both cytotoxic and helper T-cell functions. PGE$_2$ is thought to suppress cell-mediated immune responses while enhancing humoral immune responses.[44,45] PGE$_2$ suppresses chemokine and cytokine production in humans, including IFN-γ–, TNF-α–, IL-12–, and IL-1β– mediated expression of chemokines. PGE$_2$ upregulates expression of immunosuppressive cytokines, such as IL-10 and TGF-β.[45,46] This immunosuppressive effect of PGE$_2$ was demonstrated by inhibition of normal T-cell proliferation to tumor lysates with high concentrations of PGE$_2$ (Fig. 7).

The ability of mature DCs to act as potent antigen-presenting cells is related to their production of IL-12.[47] DCs deficient of IL-12 generated in the presence of PGE$_2$ promote a Th2 response.[48] A recent study demonstrated that high concentrations of PGE$_2$ caused decreased IL-12 production via increases in IL-10 production and, therefore, decreased DC function.[18] This correlates well with our data, which clearly demonstrate increased PGE$_2$ levels in the serum and within the tumor milieu and also show increased levels of intracellular IL-10 and decreased levels of IL-12 in the T cells of the breast cancer patient population.

Thus, tumor overexpression of COX-2 via the elaboration of PGE$_2$ and other mechanisms could directly block the patient's defense mechanism against cancer and promote breast cancer growth.[49] We observed overexpression of COX-2 and PGE$_2$ and impaired T-cell and DC function in breast cancer patients. If the immune system of breast cancer patients were persistently compromised, the success of immunotherapies would be limited unless the immune system could be appropriately stimulated. Many immunotherapies for cancer treatment have been partially successful in eliciting a cellular immune response; however, this response has been downregulated by tumor-derived immunosuppressive factors.

If mediators of immune suppression, such as COX-2 and PGE$_2$, can be reduced; if co-stimulation for cytotoxic T-lymphocyte effector functions can be provided with appropriate immune-based therapy to overcome the tolerizing effects of the tumor; and, most importantly, if tumor cell proliferation can be restricted, then immunotherapy can be very effective. This study, along with other studies in the literature, provides us with an immunological rationale for using COX-2 inhibition that would reduce the PGE$_2$ levels and therefore reduce immunosuppression and tumor cell growth. COX-2 inhibition combined with immune-based therapy that would induce cytotoxic T-lymphocyte activity against tumor cells is a novel concept that needs further exploration in preclinical animal models and in clinical settings.

ACKNOWLEDGMENTS

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Therapeutic Efficacy of MUC1-Specific Cytotoxic T Lymphocytes and CD137 Co-Stimulation in a Spontaneous Breast Cancer Model

Pinku Mukherjee[a], Teresa L. Tinder[a], Gargi D. Basu[a], Latha B. Pathangey[a], Lieping Chen[b] and Sandra J. Gendler[a,*]

[a]*Mayo Clinic College of Medicine, Department of Biochemistry and Molecular Biology, Mayo Clinic Scottsdale, AZ, USA*

[b]*Mayo Clinic College of Medicine, Department of Immunology, Mayo Clinic Rochester, AZ, USA*

Abstract. To study immunology in breast tumors, we have utilized a mammary gland adenocarcinoma model in which mice develop spontaneous tumors of the mammary gland which are initiated at puberty and express a human tumor antigen, MUC1. MUC1 (CD227) is over-expressed in 90% of human breast cancers and its glycosylation status and pattern of expression in cancer cells is altered. Humoral and cellular responses to MUC1 have been reported in breast cancer patients and therefore, MUC1 is being evaluated as a target for immune intervention. This mouse model of spontaneous breast cancer allows the evaluation of anti-MUC1 immune responses at all stages of the disease. In this report, we review the model as it pertains to a) the development of the tumor, b) MUC1 expression, and the native immune responses against MUC1 as tumors progress, and c) the immune suppressive microenvironment within the developing tumor. Finally, we report our latest findings describing the therapeutic efficacy of adoptively transferred MUC1-specific cytotoxic T lymphocytes (MUC1-CTL) in these mice and discuss ways to increase their effectiveness by agonistic monoclonal antibody against CD137 T cell costimulatory molecule.

INTRODUCTION

Breast cancer remains a major health problem, accounting for approximately 40,000 deaths each year in the United States. Interest is high in developing non-toxic therapeutic approaches to complement toxic surgical and chemotherapeutic strategies. The recent molecular identification of tumor antigens recognized by cytotoxic T cells (CTLs) derived from cancer patients has initiated a new era in tumor immunology. MUC1 (CD227) is a cell-associated mucin that is de-

velopmentally regulated and aberrantly expressed by more than ninety percent of breast carcinomas [1–3]. The recent description of MUC1 as a target for CTLs has raised interest in using this protein as a target for immunotherapy.

MUC1 is a transmembrane protein that exists as a large extended rod protruding from the cell membrane into the lumen of ducts and glands. The core protein consists mainly of a twenty amino acid sequence repeated from 30–90 times. These tandem repeats (TR) serve as the scaffold for O-linked oligosaccharides that cover the polypeptide core. In cancer, MUC1 expression is greatly increased on cells and in circulation, its expression is no longer restricted to the apical cell surface, and its glycosylation is altered. Both cellular and humoral immune responses to MUC1 have been reported (reviewed in [4]). However, these responses

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*Corresponding author: Sandra J. Gendler, Mayo Clinic College of Medicine, Dept. of Biochemistry and Molecular Biology and Tumor Biology Program, Mayo Clinic Scottsdale, 13400 E. Shea Blvd., Scottsdale, AZ 85259, USA. Tel.: +1 480 301 7062; Fax: +1 480 301 7017; E-mail: gendler.sandra@mayo.edu.

are not sufficiently strong to stimulate CTL killing, as most breast adenocarcinomas express MUC1 and these tumors still progress. Thus, there is a need for studies to devise effective presentation of MUC1 immunogens to stimulate immune cells to kill tumor cells. The mouse has not been a suitable preclinical model for testing vaccines, as human MUC1 differs in sequence from mouse Muc1 and is a foreign antigen in the mouse. (Human MUC1 is designated MUC1, mouse as Muc1.) We have developed MUC1-expressing mice that spontaneously develop mammary gland tumors for use in pre-clinical studies. This model effectively mimics the human situation and provides a powerful system in which to study tolerance and inactivation of CTLs in the tumor microenvironment.

Bitransgenic Mice Develop Spontaneous Mammary Gland Cancer Accompanied by Lung and Bone Marrow Metastases

Human MUC1 transgenic mice (MUC1.Tg) were bred with mice carrying the MMTV-driven polyoma middle T antigen (MT) to create MMT mice [5–7]. MUC1.Tg mice carry the full-length human MUC1 gene driven by its own promoter; they express normal levels of MUC1 in a tissue specific manner and are therefore not a model of MUC1 over expression [8]. These mice exhibit T and B cell tolerance to the MUC1 antigen, thus providing an excellent model system where MUC1-specific therapy can be studied in the context of immune tolerance [5,9] In the MMT mice, mammary gland tumors are induced by the action of a potent tyrosine kinase activity associated with the polyoma virus middle T antigen driven by the mouse mammary tumor virus long terminal repeat (MMTV) [10]. Middle T specifically associates with and activates the tyrosine kinase activity of a number of c-src family members, eliciting tumors when a threshold level of gene product has been attained. This promoter is transcriptionally active throughout all stages of mammary gland development and results in widespread transformation of the mammary epithelium and the rapid production of multifocal mammary adenocarcinomas. Hyperplastic alveolar nodules (HANs) can be detected by whole mount as early as 21 days and palpable mammary gland tumors are detectable from approximately 49 days onwards (a schematic representing the model and the tumor progression is shown in Fig. 1). Tumor progression is quite rapid, reaching 10% of body weight by about 24 weeks [6]. 100% of the female MMT mice get tumors. Tumors arise with synchronous kinetics

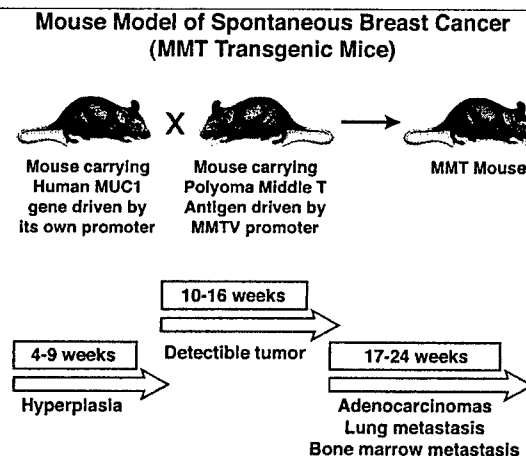### Mouse Model of Spontaneous Breast Cancer (MMT Transgenic Mice)

Fig. 1. Schematic representation of a mouse model of spontaneous metastatic breast cancer and approximate time-line of tumor progression from hyperplasia to adenocarcinomas and metastasis.

and are highly fibrotic with dense connective tissue separating individual nests of tumor cells, a pathology that closely resembles scirrhous carcinomas of the human breast [11]. Lung and bone marrow metastases were detected in MMT mice by 4 months of age [6]. Bone marrow metastasis was determined by staining bone marrow cells from MMT mice with pan-cytokeratin and MUC1, markers commonly used to detect epithelial tumor cells. About four percent of bone marrow cells were positive for both pan-cytokeratin and MUC1 by flow cytometry [6]. It is important to note that mortality in human breast cancer patients is directly associated with lung and bone metastasis. The MMT mouse is, therefore, an appropriate model for human cancer and allows us to study the effects of self-tolerance, immunity and auto-immunity to MUC1 as mammary tumors develop spontaneously.

MUC1 Expression Increases as MMT Tumors Progress

Tumor sections from 6, 12 and 20 week old non-immunized MMT mice show strong heterogeneous expression of MUC1 as tumors progress (Fig. 2). As compared to normal mammary gland, tumors express higher levels of MUC1 with increase in expression as tumors progressed from 6 weeks to 18 weeks (Fig. 2). MUC1 staining was determined using two antibodies, one recognizing the cytoplasmic tail of MUC1 (CT2) and the other recognizing the tandem repeat (TR) epitope of MUC1 (B27.29). CT2 antibody recognizes both mouse and human cytoplasmic tail (CT) of MUC1. It

is a monoclonal antibody raised in Armenian hamster. B27.29 is a mouse monoclonal reactive only against human MUC1 [12,13]. The staining pattern for B27.29 and CT2 was similar, hence, only B27.29 is shown in Fig. 2. MUC1 was expressed throughout the cytoplasm and around the cells in a pattern similar to that observed in human breast carcinomas. Thus, mammary gland tumors that occurred spontaneously in the MMT mice over-expressed the transgene protein, MUC1, in a manner similar to humans.

Cellular and humoral responses are not evidenced in MMT mice as tumors progress

In some breast cancer patients, humoral and cellular immune responses against MUC1 have been demonstrated. Thus, we determined native cellular and humoral immune responses in the non-immunized MMT mice as tumors progressed. At different stages of the tumor (6, 14, and 21 weeks), T cells were examined for expression of early and late activation markers (CD69 and CD25), as well as for expression of intracellular cytokines including IFN-?, IL-2, IL-4, IL-5, and IL-10 in response to MUC1. No significant anti-MUC1 immune responses were evident in MMT mice as tumors progressed. Furthermore, we were unable to detect presence of T cells recognizing H-2D^b/MUC1 tetramer nor did we detect precursor cytotoxic T lymphocytes (CTLs) against MUC1 at any stage during tumor progression. Dendritic cell (DC) maturation status as determined by B7 co-stimulatory receptor expression and IL-12 production also remained unchanged as the tumor progressed. With regards to humoral immune response, circulating antibody to MUC1 was undetectable by specific ELISA at any time during tumor progression. These data taken together clearly indicate that naturally occurring cellular or humoral immune responses in non-immunized MMT mice were non-detectable [6]. This lack of detectable anti-MUC1 immune response in the MMT mice implies that the immune tolerance to MUC1 is not broken by aberrantly expressed tumor-specific MUC1. In general, immune tolerance to a particular self-antigen can be broken if the immune cells encounter large amounts of the antigen systemically. In some human breast cancers, a low level anti-MUC1 immune response is generally associated with high levels of shed MUC1 in the serum which is not the case in MMT mice.

Increased Levels of MUC1 in the Serum Determine if MUC1-Specific Immune Responses will be Elicited

As tumors progressed in the MMT mice, MUC1 serum levels increased only slightly as compared to age matched MUC1.Tg mice with maximum reaching to 1500–2500 Units/ml of serum at 24 weeks of age. In age matched female MUC1.Tg mice, serum MUC1 levels ranged from 500 to 1200 Units/ml, which is likely to depend upon their estrous cycle status. The low levels of circulating MUC1 may explain the lack of an immune response to MUC1 in non-immunized MMT mice. When MMT mice were immunized with liposomal MUC1-TR and human recombinant interleukin-2, we observed significant increase in the levels of serum MUC1 as compared to untreated MMT mice. This increase in serum MUC1 directly corresponded to the increased CTL activity in these mice [6]. These results suggested that high levels of circulating tumor antigen, MUC1, may activate MUC1-specific CTL that are capable of specifically lysing MUC1-expressing tumor cells *in vitro*. We also detected low levels of circulating antibodies to MUC1 in the immunized mice suggesting that the high level of circulating tumor-associated MUC1 has changed the antigenic profile and elicited a moderate level of humoral response to MUC1. Antibodies reactive with MUC1 have been reported in a small percent of breast cancer patients [14,15]. Although the effectiveness of a humoral immune response against solid tumors is not established, it once again parallels that observed in humans. Taken together, these data demonstrate that as observed in humans, immunization strategies elicited MUC1-specific CTLs which were unable to kill the spontaneously arising breast tumors. These CTLs were found to kill MUC1$^+$ tumor cells *in vitro*, secrete IFN-γ, and express perforin and granzyme B. Despite the presence of mature functional CTLs, these mice grew tumors [6,16]. We therefore postulated that the growing MMT tumor cells evaded immune recognition and killing, a phenomenon that is becoming increasingly critical to consider in designing future immune-based therapies.

Characteristics of the MUC1-Specific CTLs

To test whether MUC1-specific CTLs enter the mammary tumor bed and are active within the tumor microenvironment, we adoptively transferred the cytolytically active MUC1-specific CTL clone into MMT mice [17,18]. These MUC1-specific CTL clone were generated from a CTL line that was originally derived
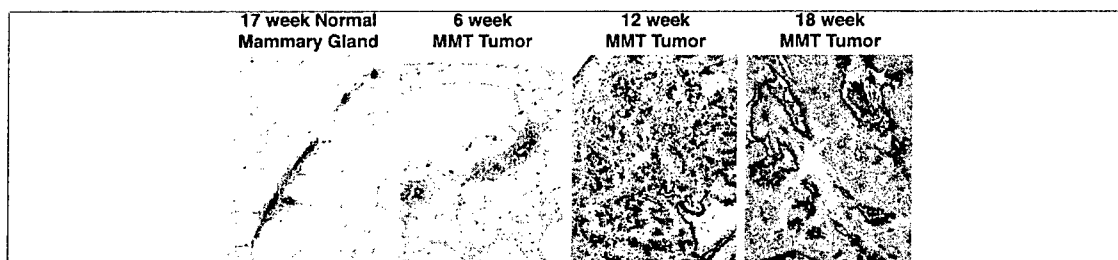
Fig. 2. MUC1 expression increases as tumors develop in MMT mice. Methacarn fixed and paraffin-embedded sections of mammary gland tumors from 6, 12 and 18 week old MMT mice were stained with B27.29, a monoclonal antibody reactive with MUC1 TR. B27.29 antibody is directly conjugated to horse radish peroxidase (HRP). Specific staining was observed on lumenal surface of mammary epithelial cells and staining pattern is similar to that seen in humans. Increase in MUC1 expression is observed as tumors developed from 6 weeks to 18 weeks. As control, normal mammary gland from 17-week old MUC1.Tg mice was used. MUC1 expression is restricted to the apical surface in normal glands as compared to tumors and the staining is less intense than in MMT tumors. Images were captured at 200X magnification.

from a MUC1-expressing pancreatic cancer mouse model that develops spontaneous tumors of the pancreas [17]. Unlike the MMT model, these mice naturally developed MUC1-specific CTLs as the pancreatic tumor progressed. The MUC1-specific CTL lines are CD8+ T cells that recognize several of the MUC1-derived peptides (Fig. 3(A)). Fourteen different MUC1 TR 9 mer peptide sequences and one MUC1 cytoplasmic tail (CT) 17 mer peptide were used to determine epitope recognition. Dendritic cells were pulsed with these peptides prior to use as targets for the CTL line and CTL clone. The line recognizes several of the MHC class I-restricted MUC1 TR peptides and the CT peptide as illustrated in Fig. 3(A). Thus, the CTL line is comprised of a heterogeneous population of T cells, expressing predominantly T cell receptor (TCR) Vβ5, 11, 13 and 2 and Vα8, 3, 11, and 2. The CTL clone, on the other hand, recognizes only a H-2D^b MHC class I-restricted immunodominant epitope of MUC1 (APGSTAPPA) and expresses Vβ5 and Vα2 [18]. When CTL line were adoptively transferred (intravenously) into MUC1.Tg mice prior to challenge with either the B16 melanoma cells expressing MUC1 transgene (B16.MUC1), or the C57 mammary tumor cells expressing MUC1 (C57mg.MUC1), the mice were protected against both types of tumor cell challenge and furthermore, developed memory T cells that could be transferred to protect naïve MUC1.Tg mice from further tumor challenge [17,18]. These MUC1-specifc CTL clones expressed high levels of perforin and granzyme B as determined by confocal microscopy and flow cytometry. Perforin and granzyme B are cellular components of cytolytic granule of CTLs and NK cells that mediates lymphocyte-dependent killing and low expression of these proteins on CTLs suggest weak cytotoxicity [19,20]. Figure 3(B) shows a representa-

tive flow cytometric dot plot of CTLs stained with anti-granzyme B antibody that was directly conjugated to phycoerythrin (PE). Almost 100% of the cells express high levels of intracellular granzyme B (Fig. 3(B)). Similarly, CTLs stained with a specific perforin antibody showed high intensity of intracellular staining by confocal microscopy (Fig. 3(B)), thus, confirming the high cytolytic activity of the MUC1-specifc CTLs.

MUC1-Specific CTLs Delay Tumor Growth in MMT Mice

To test the efficacy of the MUC1-specific CTL in MMT mice, we determined if the CTLs could enter the mammary tumor bed and whether they remained active within the tumor micro-environment. We adoptively transferred cytolytically active MUC1-specific CTL clones into 14-week-old MMT mice, which had palpable tumors. To determine if the infused CTLs could home to the tumor site, we labeled the CTL clone with carboxyfluorescein succinimidyl ester (CFSE, an *in vivo* tracking dye) prior to adoptive transfer of 1 $\times$ 10^7 CTLs intravenously. We observed that CFSE-labeled CTL home to the tumor site and lymph node by 2 days post infusion and undergo proliferation as evidenced by the discrete population of cells with distinct CFSE staining intensities. CTLs were detected up to 20 days post infusion (Fig. 4). Few to no CTLs were detected within the thymus or the spleen. To determine if the infused CTLs remained active within the tumor environment, we infused 3 week-old MMT mice with unlabeled 1 $\times$ 10^7 CTLs intravenously and boosted every 3 weeks with the same. Final infusion was given at 15 weeks and at 17 weeks of age, the mammary tumors were removed, tumor-infiltrated lymphocytes (TILs) were isolated and sorted for the infused CD8+ CTL
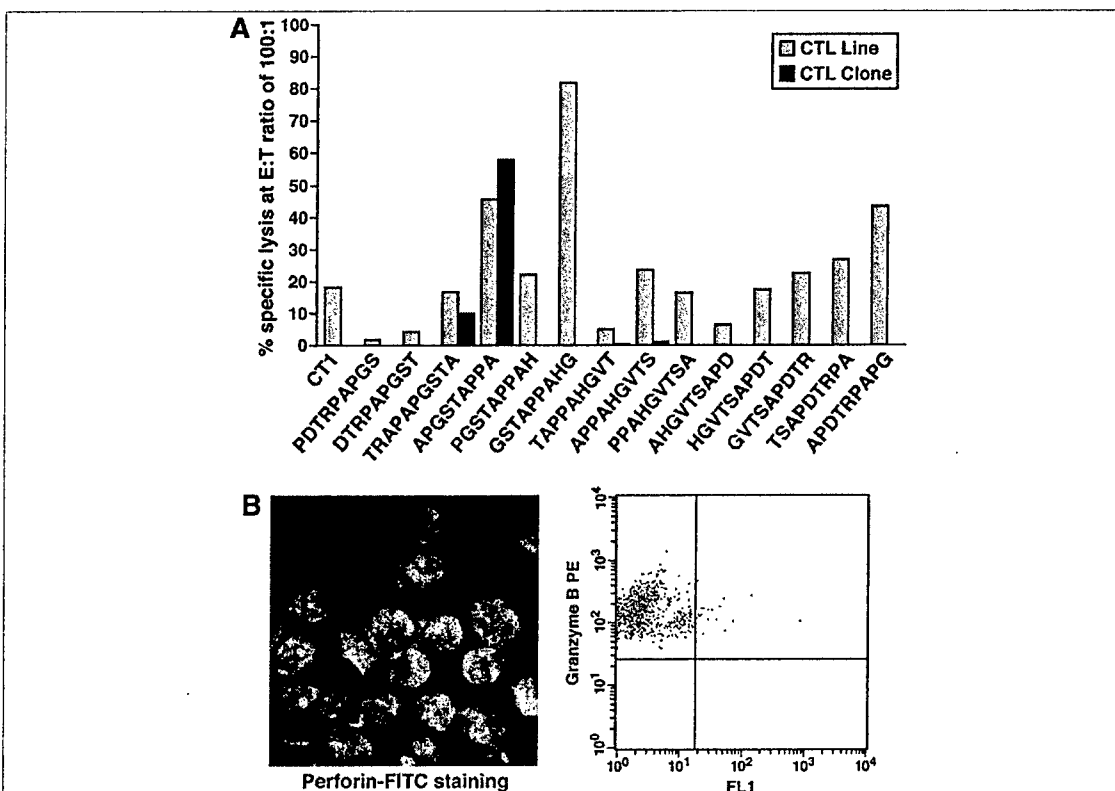
Fig. 3. A. Epitopes recognized by MUC1-specific CTL line and clone. Fourteen different MUC1 TR 9 mer peptide sequences and a MUC1 cytoplasmic tail 17 mer peptide were used to determine epitope recognition. Dendritic cells were pulsed with the peptides at 10(-6)M concentration prior to use as targets for the CTL line and CTL clone. A standard 8hr ^{51}Cr-release assay was performed. Specific lysis was performed with several effector to target (E : T) ratios. Figure shows data from 100:1 effector to target ratio. Recognition of the peptides by CTLs is gauged by the percent of specific lysis. Lysis at and above 18–20% is thought to be significant. Thus, MUC1 line consists of several CTL populations that recognize eight different TR peptides and the CT peptide. MUC1 clone recognizes only APGSTAPPA peptide. Degree of lysis directly correlates to the affinity by which the peptide is recognized by the CTL. CTLs seem to recognize APGSTAPPA and GSTAPPAHG with the highest affinity. B. MUC1-specific CTLs express high levels of perforin and granzyme B. CTLs were permeabilized using the Pharmingen Permeabilization kit and stained with either perforin-FITC antibody (Alexis Biochemicals, San Diego, CA) and examined by confocal microscopy (left panel); or with granzyme B antibody directly conjugated to PE (Caltag Laboratories, Burlington, CA) and analyzed by flow cytometry (right panel).

clone based on their CD8 expression and TCR Vβ5 expression (Fig. 5(A), 2nd panel). TILs isolated from age matched MMT mice that were not infused with CTLs were used as controls (Fig. 5(A), 4th panel). The CTL clone maintained *in vitro* with the same profile as the sorted TILs were used as positive control (Fig. 5(A), 1st panel). The sorted cells were used in three assays to determine: 1) if these cells were hypo-responsive to MUC1 and therefore were tolerant to MUC1 antigen, 2) if these cells maintained their cytotoxicity after encounter with the tumor cells *in vivo*, and 3) if the sorted CTLs expressed granzyme B, a protein needed for the CTLs to be functionally lytic. In Fig. 5(B), we clearly show that in contrast to the CTL clone,

the sorted Vβ5^{+}/CD8^{+} TILs had significantly reduced proliferation in response to MUC1 presented on irradiated DCs, suggesting that the infused CTLs become tolerized to tumor antigen MUC1 after encounter with the growing mammary tumor cells. Similarly, the sorted Vβ5^{+}/CD8^{+} TILs were unable to kill tumor cells expressing MUC1 *in vitro*, while the same cells that had not encountered tumor cells and were maintained in tissue culture remained highly cytolytic (Fig. 5(C)), suggesting that the infused CTL became cytolytically nonfunctional in the tumor environment. This cytolytic capability of the sorted TILs was further confirmed by the observation that the sorted Vβ5^{+}/CD8^{+} TILs from the tumors showed significantly lower levels of granzyme
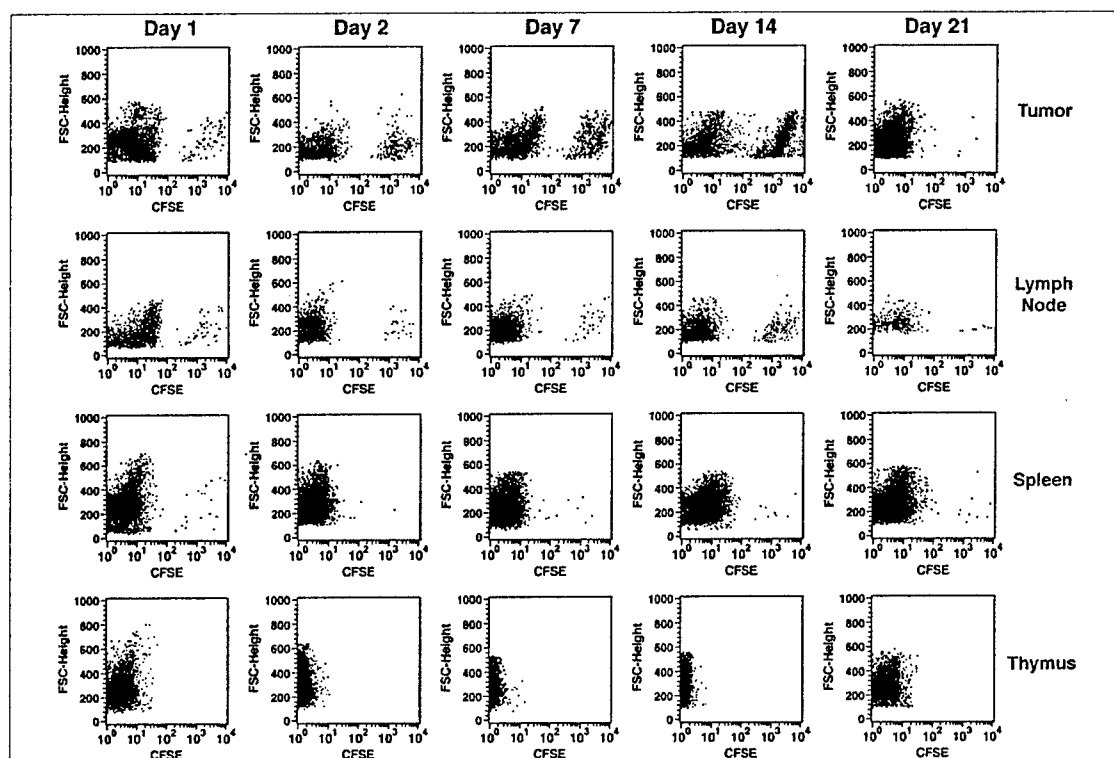
Fig. 4. CFSE labeled MUC1-specific CTL home to the lymph node and mammary gland tumor. CTL clone were stained with carboxyfluorescein diacetate succinimidyl ester (CFSE). Cells were incubated with 2 ul of 5 mM CFSE per 1×10^7 cells/ml in PBS-0.1%FBS for 10minutes at 37°C and quenched by adding 5 times the volume of ice-cold media. 2×10^7 CFSE-stained CTLs were injected intravenously into 14-week old tumor bearing MMT mice. At various times post CTL infusion, TILs, lymph node cells, spleen cells and thymic cells were isolated and monitored for CFSE staining by flow cytometry. $n = 5$ mice and a representative dot plot is shown. By 21 days post infusion, CFSE labeled cells were undetectable.

B staining as compared to the CTLs that were maintained in culture (Fig. 5(D)). These findings are extremely significant, as immunotherapy can now be designed to reverse this established tolerance or prevent the induction of tolerance. The treatment group that includes CTL + CD137 monoclonal antibody (mAb) is represented in this Figure, but the experiment is described in a later section.

Nevertheless, when CTLs were continuously infused in MMT mice starting at 3 weeks of age, we observed a significant reduction in tumor growth and progression (Fig. 6). Clearly, adoptive transfer of MUC1-specific CTL early during tumorigenesis can reduce tumor burden and enhance survival in these mice. However, once CTL infusions were stopped, the tumors grew back. These results suggest strongly that immunotherapy can work if the tumor microenvironment is somehow altered. For example, if mediators of tumor-induced immune-suppression can be reduced, co-stimulation

for CTL function can be provided to overcome the tolerizing effects of the tumor microenvironment, and most importantly, if tumor-cell proliferation can be restricted.

Immunosuppressive Tumor Microenvironment in the Mammary Gland Tumor

Since infused CTLs became tolerant and cytolytically non-functional within the tumor microenvironment, we evaluated the presence of immunosuppressive mediators in mammary tumors. Data revealed that the tumor cells secrete IL-10 and TGF-ßthat are partly responsible for the down-regulation of CTL activity [6]. IL-12 production and expression of co-stimulatory receptors by DCs was also found to be reduced, suggesting sub-optimal antigen presentation within the tumor micro-environment. In addition, tumor cells down-regulated surface major histocompati-
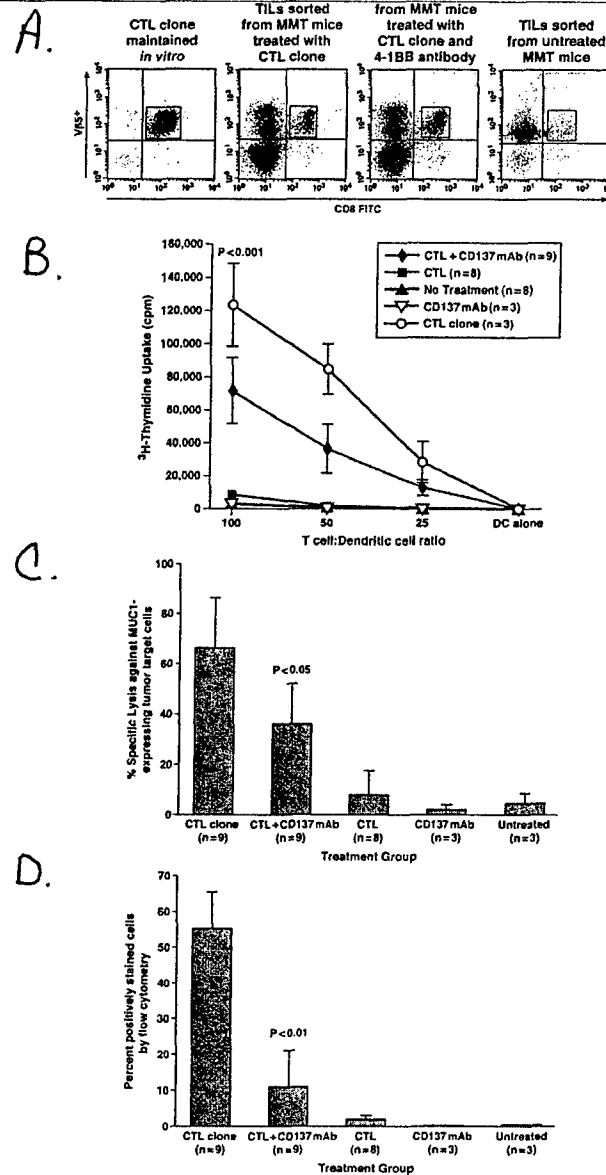
Fig. 5. Adoptively transferred CTL become tolerant to MUC1 antigen and are cytolytically inactive within the tumor microenvironment. TILs were isolated from tumors of MMT mice that received adoptively transferred MUC1-specific CTL clone ($V\beta5^+$/CD8$^+$ T cells). CD137 mAb was injected every week at 100 ng/mouse/100ul intraperitoneally. A) Flow cytometric profile of TCR $V\beta5^+$/CD8$^+$ T cells sorted from TILs by flow cytometry. (box represents the population that was sorted). Profile of untreated MMT and MMT treated with CD137 mAb alone looked identical and therefore we chose to show only one group. B) Proliferation by 3H-thymidine uptake of the sorted T cells. Proliferation was determined in response to 25 mer MUC1 peptide (STAPPAHGVTSAPDTRPAPGSTAPP) presented on dendritic cells. C) Cytotoxic activity by 51Cr-release assay of sorted T cells against MUC1-expressing B16.MUC1 melanoma tumor cells. As positive controls, CTL clone maintained *in vitro* was used and as negative controls TCR $V\beta5^+$/CD8$^+$ T cells from mammary tumor of age matched untreated MMT mice was used. D) Granzyme B staining of sorted T cells by flow cytometry. Cells were permeabilized and antibody to Granzyme B directly conjugated to PE from B.D. Pharmingen was used at 1 ug/10^6 cells to determine intracellular staining using flow cytometry. Treatment groups include: MMT treated with CTL; MMT treated with CTL + CD137 mAb; MMT treated with CD137 mAb; and untreated MMT. p-values are shown in the figure and represent significant differences between CTL + CD137 group as compared to untreated or α–CD137 alone or CTL alone groups. 6 mice were enrolled in the α–CD137, however, 3 mice were found morbid prior to end of experiment and were removed from the study.
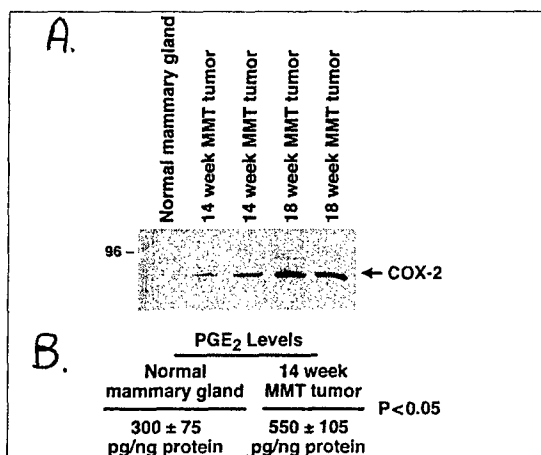
Fig. 7. MMT tumors express COX-2 and the downstream product PGE2. A) Western blot analysis of mammary tumor lysate derived from 14 and 18-week old MMT mice compared to normal mammary gland lysate. SDS-PAGE electrophoresis was performed using 12% resolving gel. 50 μgs of protein was loaded per lane. Gels were immunoblotted and probed for COX-2 with specific COX-2 monoclonal antibody (goat polyclonal, clone C20, Santa Cruz Biotechnology Inc., Santa Cruz, CA) at 1:200 dilution. MMT tumors over express COX-2 as tumors progress. B) PGE2 levels determined by specific ELISA in 14-week old MMT tumor lysate as compared to normal mammary gland lysate (n = average of 6 mice). Levels in the lysates were determined using a specific ELISA kit for PGE2 (Cayman Pharmaceuticals, Ann Arbor, MI). Manufacturer's recommended protocols were followed. Compared to normal mammary gland, PGE2 in tumor is significantly higher ($p < 0.05$).

bility complex (MHC) class I molecules to avoid immune recognition as well as expressed higher levels of $CD4^+CD25^+$ T regulatory cells [6,16]. Recently, we have found over expression of COX-2 in the MMT tumors by western blot analysis (Fig. 7) as well as by immunohistochemistry (data not shown). COX-2 is an inducible enzyme that is over expressed in many tumors and is involved in many aspects of tumorigenesis. COX-2 converts arachidonic acid to prostaglandins, especially prostaglandin E2 (PGE2), a well-characterized immune cell suppressor [21–24]. In MMT mice, we found significantly higher levels of serum PGE2 as compared to normal mouse sera, suggesting that the COX-2/PGE2 pathway may be partly responsible for the immune suppressive tumor micro-environment in MMT mice. We have recently shown that T cell and DC functions in newly diagnosed breast cancer patients are impaired and that over expression of COX-2 and PGE2 may play a significant role in inducing such suppression [25]. Together, these data indicate that mammary gland tumor cells utilize a variety of immune evasion mechanisms to avoid CTL killing. Managing all

of these immune evasion pathways seems formidable. On one hand, we have immune responses against the tumor and, on the other hand, there is tumor-induced immune suppression. Tilting the balance towards more sustained and increased CTL activity may be easier to accomplish than addressing every one of the tumor evasion mechanisms. One way to accomplish this is by providing the activated CTLs with appropriate co-stimulation to increase their efficiency in killing tumor cells.

CD137 mAb can Reverse Tolerance in vivo in MMT Mice and has a Synergistic Anti-Tumor Effect when Combined with MUC1-Specific CTL Therapy

The CD137 glycoprotein is a member of the tumor necrosis factor receptor superfamily expressed on primed but not on naïve $CD4^+$ and $CD8^+$ T cells. CD137 binds to a specific ligand (CD137L) expressed on several antigen presenting cells (APCs) and signals either through ligand binding or by specific agonistic antibody to deliver a dual mitogenic signal for further T cell activation and proliferation. It has been shown that administration of CD137 mAb can amplify T cell-mediated immune responses and can eradicate established tumors [26,27]. Recent experiments suggest that anti-tumor effect of CD137 antibody is to reverse T cell tolerance/anergy [28] that is so often induced by tumor cells. Thus, we evaluated the efficacy of CD137 antibody therapy in reversing tolerance in our *in vivo* breast cancer model, since we have already established that adoptively transferred CTLs become tolerized to MUC1 and are cytolytically non-functional within the tumor microenvironment. We were therefore able to specifically answer whether treatment with CD137 antibody could reverse this tolerizing effect within the mammary gland tumor. MMT mice were infused with 1×10^7 CTLs starting at 3 weeks of age and boosted every 3 weeks with the same. Final infusion was given at 15 weeks and TILs were sorted for $CD8^+V\beta5^+$ T cells at 17 weeks (Fig. 5(A), 3rd panel). Along with the CTL infusions, these mice received CD137 mAb at 100 ug/mouse intraperitoneally every week until mice were sacrificed at 17 weeks of age. Data clearly indicate that CD137 mAb treatment was extremely effective in reversing tolerance such that the CTL clone regains responsiveness to MUC1 antigen post CD137 treatment (Fig. 5(B)). Treatment with this antibody was also effective in restoring the cytolytic activity of the CTL clone as measured by the ^{51}Cr-release assay and granzyme B staining (Figs 5(C) and (D)). Most impor-
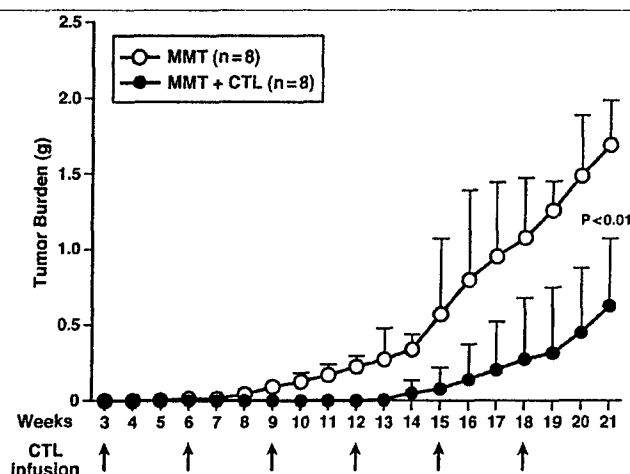
Fig. 8. Adoptively transferred MUC1-specific CTL clone inhibits tumor progression in MMT mice. MUC1-specific CTLs (2×10^7 cells i.v.) were infused into MMT mice starting at three weeks of age. Infusions were given every three weeks from week 3 onward. Six infusions were performed. Tumor growth was inhibited until the cessation of CTL injections, at which time tumors began to progress. CTL infused MMT mice showed significantly lower tumor burden as compared to untreated MMT mice ($p < 0.01$).

tantly, this reversal of CTL tolerance *in vivo* translated well to anti-tumor response. When mice were sacrificed at 17 weeks, tumor burden was evaluated. MMT mice treated with CTL alone had a tumor burden of 0.5 g that was significantly lower than untreated control mice with 0.9 g tumor ($p < 0.05$) or mice treated with CD137 mAb alone with 1 g tumor. Treatment with CD137 increased the effectiveness of CTLs since MMT mice treated with CTL and CD137 mAb had a tumor burden of 0.2 g ($p < 0.01$) (Fig. 8). The unresponsiveness of MMT tumors to CD137 mAb alone can be explained by the fact that CD137 is only expressed on activated T cells and does not work on naïve, non-primed T cells [29] and untreated MMT mice have no detectable primed T cells. Although 6 mice were enrolled in the anti-CD137 treatment arm, 3 mice were found morbid prior to end of experiment and were removed from the study. These results demonstrate that MUC1-specific CTLs are very effective against spontaneously arising MUC1+ breast tumors and that their efficacy can be increased and maintained for longer time with appropriate co-stimulation such as with anti-CD137 antibody. Lung metastasis was not evaluated in these mice, since tumor burden at 17 weeks even in the untreated mice is not large enough for lung metastasis to develop. The next set of experiments will determine the effect such a treatment on lung and bone marrow metastasis.

CONCLUSIONS

We have shown that the spontaneous mammary gland tumors that arise in the transgenic MMT mice appropriately models the human metastatic breast cancer. Several features of the tumor resemble the human disease, for example, the tumors arise in an immune competent host within the context of MUC1-tolerence; the tumors aberrantly over express MUC1, the tumors metastasize to the lungs and the bone marrow, and very little to no immune response against the tumor antigen, MUC1, is evident in the model. As observed in humans, treatment with anti-MUC1 immunotherapy leads to a robust cellular immune response accompanied with a moderate humoral immune response against the immunizing antigen, MUC1, which does not translate efficiently to a clinical response. We further show that the inefficiency of MUC1-specific CTLs to affect tumor burden and survival is partly due to the immunosuppressive tumor microenvironment that renders the infiltrating CTLs inactive with regards to antigen recognition and killing. Similar to observations in human breast cancer, several immunosuppressive factors were identified in the MMT tumor microenvironment. In fact, in human disease, immunosuppression has not only been described within the tumor microenvironment but also systemically. Finally, we show in our model, that the most effective anti-tumor response was generated when mice were treated with continuous intravenous infusions of MUC1-specific CTLs and that the CTL
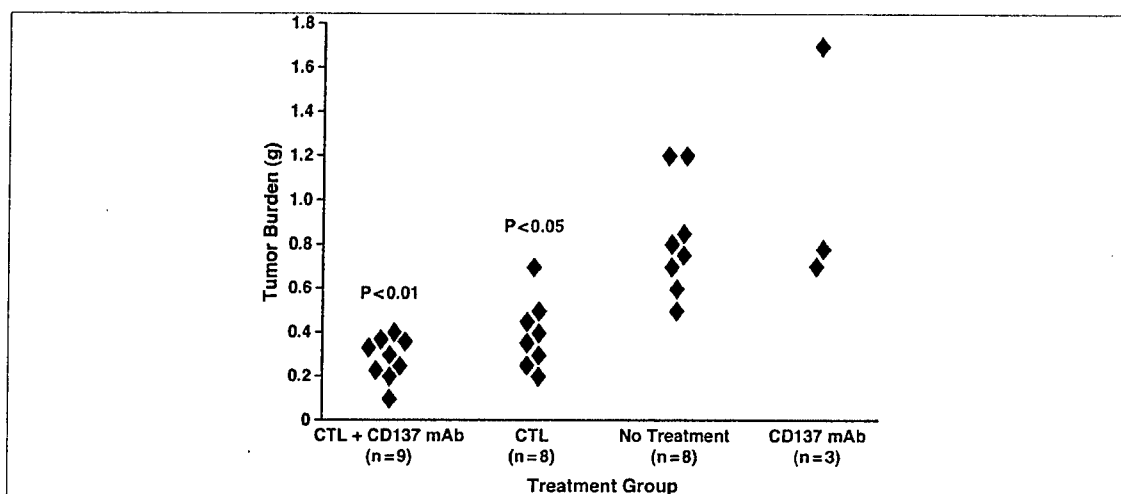
Fig. 8. CD137 mAb in combination with MUC1-specific CTL therapy is more efficient in reducing tumor burden than CTL therapy alone. MMT mice were injected with MUC1-specific CTLs (2×10^7 cells i.v.) every three weeks starting at 3 weeks; and CD137 mAb was injected at 100 ug/mice (i.p.) every week starting at 6 weeks. Individual animal data is shown as scatter plot. P values indicate significant differences between treatment groups and untreated control mice. Tumor burden was significantly lower in CTL + CD137 mAb treated mice ($p < 0.01$) and in CTL treated mice ($p < 0.05$) as compared to untreated and CD137 mAb treated mice. 6 mice were enrolled in the CD137 mAb group; however, 3 mice were found morbid prior to end of experiment and were removed from the study. CD137 mAb, clone 2A, was raised in rats against a fusion protein consisting of the extracellular domain of murine CD137 and the human immunoglobulin C region [26].

efficiency was significantly enhanced when treatment was combined with CD137 co-stimulation. From our data, we suggest that one of the mechanisms by which CD137 co-stimulation increases CTL activity is by reversing tumor-induced CTL tolerance and hence inactivity *in vivo*. These results allow us to propose that such a therapy may be beneficial for the treatment of metastatic breast cancer in humans.

ACKNOWLEDGEMENTS

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COX-2 inhibitor induces apoptosis in breast cancer cells in an *in vivo* model of spontaneous metastatic breast cancer

Gargi D. Basu[#], Latha B. Pathangey[#], Teresa L. Tinder, Michelle LaGioia, Sandra J. Gendler[1],

and Pinku Mukherjee

Mayo Clinic College of Medicine, Department of Biochemistry and Molecular Biology and

[1]Tumor Biology Program, Scottsdale, AZ

[#] Both authors contributed equally to this work

Corresponding Author
Pinku Mukherjee
Mayo Clinic College of Medicine
13400 E. Shea Blvd.
Scottsdale, AZ 85259
(480) 301-6327
Fax – (480) 301-7017
email: mukherjee.pinku@mayo.edu

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ABSTRACT

Cyclooxygenase-2 (COX-2) inhibitors are rapidly emerging as a new generation of therapeutic drug in combination with chemotherapy or radiation therapy for the treatment of cancer. The mechanisms underlying its anti-tumor effects are not fully understood and more thorough preclinical trials are needed to determine if COX-2 inhibition represents a useful approach for prevention and/or treatment of breast cancer. The purpose of this study was to evaluate the growth inhibitory mechanism of a highly selective COX-2 inhibitor, celecoxib, in an *in vivo* oncogenic mouse model of spontaneous breast cancer that resembles human disease. The oncogenic mice carry the polyoma middle T antigen (MTag) driven by the MMTV promoter and develop primary adenocarcinomas of the breast. Results demonstrate that oral administration of celecoxib caused significant reduction in mammary tumor burden associated with increased tumor cell apoptosis and decreased proliferation *in vivo*. *In vivo* apoptosis correlated with significant decrease in activation of protein kinase B, Akt, a cell-survival signaling kinase, with increased expression of pro-apoptotic protein, Bax, and decreased expression of anti-apoptotic protein, Bcl-2. In addition, celecoxib treatment reduced levels of pro-angiogenic factor, vascular endothelial growth factor (VEGF), suggesting a role of celecoxib in suppression of angiogenesis in this model. Results from these preclinical studies will form the basis for assessing the feasibility of celecoxib therapy alone or in combination with conventional therapies for treatment and/or prevention of breast cancer.

INTRODUCTION

In the United States, breast cancer is the second most common cancer and contributes to 40,000 deaths in a year. If confined within the breast, the tumor can be surgically removed with an increased survival rate. However, primary tumors that metastasize to distant sites such as lymph nodes, lungs, liver and brain correlate with poor prognosis. Complications from metastatic disease are the leading cause of cancer-related deaths. Mean survival for patients with metastatic breast cancer is 18-24 months. Response to chemotherapy or endocrine therapy in metastatic breast cancer patients is ~50% (1). Clearly, a need for development of novel therapies to enhance the existing triad of surgery, radiation and chemotherapy is evident. COX-2, the inducible form of the COX enzymes, catalyzes conversion of arachidonic acid to prostaglandin H_2 (PGH$_2$), which is further converted to several other prostaglandins with diversified functions. Deregulation of COX-2 activity and downstream prostaglandins play a vital role in carcinogenesis, inflammation, and tissue damage (2-5). COX-2 is over expressed in many cancers including breast cancer, and the major functional prostaglandin in breast cancer is prostaglandin E_2 (PGE$_2$). Over expression of COX-2 protein and PGE$_2$ during carcinogenesis is implicated in proliferation, invasion, apoptosis, immune-suppression and angiogenesis. COX-2 is induced by a variety of factors including tumor promoters, cytokines, growth factors and hypoxia. Importantly, selective inhibition of this enzyme reduces adenocarcinoma formation and cancer progression in preclinical animal models (6-8). The first direct evidence of COX-2 function in cancers came from the study by Eberhart et al. (9) documenting significant elevations in COX-2 expression in 85% of human colorectal carcinomas and 50% of colorectal adenomas. COX-2 overexpression has since been found in many other human cancers including breast (10,

11), esophageal (12, 13), lung (14, 15), prostate (16, 17), bladder (18, 19), skin (20, 21), and pancreas (22, 23).

Studies with specific inhibitors of COX-2 enzyme have shown significant effects in reducing the incidence and progression of tumors in both animal models and in treatment of cancer patients (6-8, 24-30). Studies to evaluate effects of COX-2 specific inhibitors in the treatment of breast cancer have started recently and therefore data are limited. In animal studies, COX-2 inhibitors have shown promising results. In rat models of chemical carcinogenesis, COX-2 inhibitors significantly reduced incidence and size of mammary tumors (31, 32). COX-2 inhibitors were also effective in retarding tumor progression and metastasis in mouse models of injected breast cancer cell lines as well as in xenograft models of human breast cancer cells in nude mice (24, 33, 34). Clinically, COX-2 inhibitors have been used in combination with other anti-cancer drugs or radiation therapy to treat solid tumors, mostly focusing on colon and colorectal cancers. Reports emerging from these studies strongly suggest that COX-2 inhibitors may emerge as a new generation of therapeutic drugs for cancer therapy. A recent report indicated that regular NSAID use for 5-9 years was associated with a 21% reduction in the incidence of breast cancer and regular use for more than 10 years was associated with 28% reduction (35). This area of research is under-explored and more thorough pre-clinical trials are needed to further determine if COX-2 inhibition represents a useful approach to treatment of breast cancer.

Preclinical studies must precede clinical trials and use of appropriate mouse models is key to the development of efficient therapeutic strategies. We have used the oncogenic mice that carry the polyoma virus middle T antigen driven by the mouse mammary tumor virus long terminal repeat

(MMTV) promoter (known as the MTag mice) in this study. These mice develop spontaneous tumors of the breast, which metastasize to the lungs and bone marrow. All mice are congenic on the C57BL/6 background to eliminate strain-specific modifier effects. In the MTag mice, mammary gland tumors are induced by the action of a potent tyrosine kinase activity associated with the polyoma virus middle T antigen driven by the MMTV promoter (36). Middle T specifically associates with and activates the tyrosine kinase activity of a number of c-src family members, eliciting tumors when a threshold level of gene product has been attained. In these mice, the MMTV promoter is transcriptionally active throughout all stages of mammary gland development, which results in widespread transformation of the mammary epithelium and the rapid production of multifocal mammary adenocarcinomas. Focal atypical lesions can be detected by whole mount as early as 21 days and palpable mammary gland tumors are detectable from approximately 60 days onwards. Tumor progression is quite rapid, reaching 10% of body weight by about 20 – 24 weeks. All of the female mice get tumors. Tumors arise with synchronous kinetics and are highly fibrotic with dense connective tissue separating individual nests of tumor cells, a pathology that closely resembles scirrhous carcinomas of the human breast (37, 38). These mice exhibit metastasis in the lungs (60%) and micro-metastasis in the bone marrow by 4 months of age (39). Therefore, the MTag mouse model is an appropriate model for human metastatic breast cancer, in which to evaluate therapeutic strategies as well as understand the mechanisms associated with therapy-induced growth inhibition. This is the first study to evaluate the efficacy and growth-inhibitory mechanisms of celecoxib in an *in vivo* model of spontaneous metastatic breast cancer.

RESULTS

Celecoxib treatment caused significant reduction in primary mammary tumor burden.
Ten-week old tumor-bearing female MTag mice were gavaged daily for four weeks with celecoxib at 5, 10 or 20mg/kg body weight. In mice, at 10 and 20mg/kg dose, the concentration of celecoxib in the plasma ranges from 6.5μM - 13μM at 2h, and 4.2μM – 8μM at 4h post celecoxib treatment (40). This dose is attainable clinically and sufficient to inhibit PGE_2 (41). At 10 weeks, mice have small palpable tumors (1-2 tumors, ~0.1 to 0.5mg tumor weight). Complete blood count analysis (CBC) including hemoglobin levels was performed to determine cytopenia and/or anemia post celecoxib treatment. Regardless of the celecoxib dose, there was no detectable change in their CBC or hemoglobin levels (data not shown) as compared to untreated MTag mice. Flow cytometric analysis of T cells, B cells and NK cells revealed no change in treated versus control MTag mice, nor were there any signs of weight loss in treated mice (data not shown). This suggested that celecoxib was well tolerated in these mice with no detectable signs of toxicity. Mice were sacrificed at 14 weeks of age, tumors removed and serum collected. Tumor burden in MTag mice treated with 10 and 20mg/kg dose was significantly reduced ($p < 0.003$ for 10mg/kg and $p<0.01$ for 20mg/kg; Figure 1). Note that in this study, we started the treatment at 10weeks when the mice had established tumors. The purpose of this study was to focus on the short-term effect of celecoxib on breast cancer cells *in vivo* at early times during tumor development, and evaluate the mechanism of action of the drug on primary breast cancer cells. All mice were terminated at 14 weeks of age.

Celecoxib induces apoptosis in breast cancer cells *in vivo*. We have recently reported that celecoxib induces growth inhibition of human and mouse breast cancer cells *in vitro* by

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simultaneously activating tumor cell apoptosis and inhibiting proliferation (42). Apoptosis of primary MTag tumor cells was determined by Annexin-V/PI staining and flow cytometry. Data revealed significant increase in apoptotic cell population at 10 and 20 mg/kg celecoxib dose as compared to control MTag mice (39% in control mice versus 65% in 10mg/kg dose, p< 0.05 and 59% in 20mg/kg dose, p< 0.05). The lowest dose (5mg/kg) did not have a significant effect (Figure 2A). Tumor cells from untreated MTag mice gave similar percent of apoptotic cells (~35-40%) as vehicle treated mice (data not shown). The high baseline apoptosis level in vehicle treated and untreated mice is likely due to the method of isolating single cells. However, the 1.5 to 1.7 fold increase following celecoxib treatment was reproducibly observed.

We also evaluated celecoxib-induced apoptosis *in situ* by detection of DNA fragmentation using the TUNEL assay (43). We observed an increase in TUNEL positive cells in celecoxib treated tumor sections *in situ* as compared to control tumor sections confirming the flow cytometry data (Figure 2B). Representative IHC images of vehicle and celecoxib treated MTag tumor sections are shown at 100X magnification demonstrating considerable TUNEL-positivity in celecoxib treated versus control MTag tumor sections.

Increased Bax and decreased Bcl-2 in tumor lysate derived from celecoxib treated MTag mice

The downstream signaling pathways involved in COX-2-induced apoptosis are not well understood, but at least three pathways have been suggested; Bcl-2 mediated pathway, the nitric oxide pathway, and production of ceramide (44). Since it has been previously shown in cell lines that celecoxib-induced apoptosis is associated with decreased Bcl-2 (an anti-apoptotic protein)

and increased Bax (a pro-apoptotic protein), we evaluated the levels of Bcl-2 and Bax by western blot analysis of whole MTag tumor lysate post celecoxib treatment. Treatment with celecoxib at 10mg/kg and 20mg/kg induced increased expression of Bax (inducer of apoptosis) in all 5 mice tested as compared to vehicle treated tumors (Figure 3A). The increase was most pronounced at the 10mg/kg dose of celecoxib. Simultaneously, there was decrease in Bcl-2 (inhibitor of apoptosis) protein expression in the 10mg/kg and 20mg/kg dose of celecoxib. (Figure 3B). Untreated MTag tumor lysate was used as control in the first lane. These tumor lysates were prepared from 21-week MTag tumors while the treated mice were at 14 weeks of age. This could explain the difference in protein expression observed between vehicle treated and untreated MTag tumors. Densitometric analysis of the western blots indicates significant increase in Bax protein levels between vehicle-treated and 10mg/kg ($p < 0.05$) and 20mg/kg celecoxib-treated tumor lysates ($p < 0.06$). Similarly, significant decrease in Bcl-2 was observed between vehicle and 5mg/kg-treated mice versus 10mg/kg ($p < 0.05$) and 20mg/kg ($p < 0.05$) celecoxib treatment groups. Thus, data suggest that celecoxib-induced apoptosis in MTag tumor cells *in vivo* is associated with an elevated expression of Bax and reduced expression of Bcl-2 proteins. These results give further credence to the flow cytometry and TUNEL data, confirming that celecoxib induces apoptosis *in vivo* in a highly aggressive and metastatic breast cancer model.

Reduced phosphorylation of Akt in tumor lysate derived from celecoxib treated MTag mice

Protein kinase B (PKB), Akt, is a serine/threonine protein kinase that is involved in promoting cell survival signals through the PI3K pathway, leading to inactivation of a series of pro-apoptotic proteins. These kinase activities are frequently deregulated in human disease including cancer (45). Akt represents a key-signaling component in cell survival by activating downstream

pro-apoptotic proteins and caspases (48-50). Celecoxib has recently been shown to induce apoptosis of cancer cells by blocking Akt activation in cultured prostate cancer cells (46, 47). Thus, to explore whether inhibition of Akt activation may be linked to the observed *in vivo* apoptosis in MTag tumors, we determined the effect of *in vivo* celecoxib administration on phosphorylation of Akt (at Ser^{473} in the carboxy terminus) in MTag tumors. Data demonstrate that celecoxib substantially suppresses phosphorylation of Akt in MTag tumors. Two out of 5 mice in the 5mg/kg dose showed reduced phosphorylation, while 4 out of 5 in 10mg/kg and 5 out of 5 mice in 20mg/kg dose showed reduced Akt activation (Figure 4). Densitometric analysis clearly indicates significant downregulation of Akt phosphorylation in celecoxib-treated tumors as compared to vehicle treated tumors ($p < 0.05$ for 10mg/kg and 20mg/kg celecoxib). All tumors showed approximately equivalent levels of the Akt protein as shown in Figure 4, lower panel. This result clearly suggested the involvement of the Akt pathway in induction of apoptosis *in vivo* in our mouse model of spontaneous breast cancer.

Celecoxib inhibits tumor cell proliferation

Anti-proliferative effect of *in vivo* celecoxib treatment was determined by *in situ* immunohistochemical analysis of MTag tumor sections stained with Proliferating Cell Nuclear Antigen (PCNA). A representative light microscope image suggests inhibition of proliferation in MTag tumors *in vivo* with 10 and 20mg/kg celecoxib treatment (Figure 5). PCNA protein levels peak during the S-phase of the cell cycle, and is almost undetectable in other phases of the cycle. Vehicle and 5mg/kg celecoxib treatment shows almost every cell expressing PCNA, indicative of highly proliferative cells (Figure 5A and B). With 10mg/kg and 20mg/kg treatment, fewer cells expressed PCNA staining, suggestive of fewer cells undergoing proliferation (Figure 5C

and D). A lymph node (LN) within an untreated mammary tumor section shows only a few proliferating cells, confirming the specificity of the stain (Figure 5E). Staining specificity is further confirmed with second antibody control (Figure 5F).

Celecoxib treatment significantly reduced serum levels of PGE$_2$ *in vivo*

Next, we analyzed sera and tumor lysate from celecoxib-treated and vehicle treated MTag mice for PGE$_2$ levels to assess COX-2 activity *in vivo*. COX-2 converts arachidonic acid to bioactive prostaglandins (PGs). It has been demonstrated that COX-2 derived PGE$_2$ is the major prostaglandin produced by breast cancer cells, and may be required for the angiogenic switch leading to initiation and progression of mammary cancer in a MMTV-COX-2 transgenic mouse model (51). Production of secreted PGE$_2$ is an appropriate measure of COX-2 activity in the MTag mouse model. PGE$_2$ is unstable *in vivo* and measurement of the metabolites is necessary to provide a reliable estimate of actual PGE$_2$ production. Thus, we measured PGE$_2$ metabolite (PGEM) using a commercially available ELISA. A significant reduction in serum PGEM is observed in 10mg/kg and 20mg/kg celecoxib treated MTag mice as compared to pretreatment and vehicle treated mice (2000pg/ml in vehicle-treated mice versus <1000pg/ml in 10mg/kg celecoxib-treated mice, p< 0.01) (Figure 6A). Similar reduction in PGEM was observed in tumor lysates (data not shown). Note that the serum PGEM levels never reached the values observed in non-tumor C57BL/6 mice of 300pg/ml (Figure 6A). This suggests that although celecoxib was partially effective in reducing PGEM levels, treatment was not sufficient to completely reverse the up regulation of PGE$_2$ levels in MTag mice since these mice were not completely tumor-free.

Celecoxib-induced apoptosis of breast tumors

To test if celecoxib had a direct effect on COX-2 protein expression in the tumor, we evaluated COX-2 protein expression in tumor lysates from vehicle and celecoxib treated-MTag tumors. MTag tumors from untreated and vehicle-treated mice expressed higher levels of COX-2 (Figure 6B) as compared to celecoxib-treated (10mg/kg and 20mg/kg) tumors, indicating that celecoxib has a direct effect on COX-2 protein expression *in vivo*. Densitometric analysis showed some difference between vehicle-treated and 10 or 20mg/kg celecoxib treated tumors ($p < 0.07$). However, the direct effect of celecoxib on COX-2 protein expression was less significant ($p < 0.07$) than its effect on COX-2 activity, as measured by PGE_2 levels.

Celecoxib treatment reduced VEGF levels *in vivo*

It has recently been shown that COX-2 induced PGE_2 stimulated the expression of angiogenic regulatory genes including VEGF in mammary tumor cells isolated from COX-2 transgenic mice and that treatment with indomethacin (non-specific COX-inhibitor) suppressed the expression of these genes *in vitro* (51). We therefore evaluated levels of *in vivo* VEGF protein levels in the tumor microenvironment of MTag tumors post celecoxib treatment. Treatment with celecoxib (10mg/kg or 20mg/kg) reduced VEGF levels in the tumor lysate in 4 out of 6 treated mice as compared to vehicle treated MTag tumors ($p < 0.05$) (Figure 7). No reduction was observed in mice treated with 5mg/kg celecoxib. Untreated MTag tumor lysate had similar levels as vehicle treated MTag mice (data not shown).

DISCUSSION

We demonstrate for the first time that *in vivo* treatment with celecoxib causes significant reduction in mammary gland tumor burden in a mouse model of spontaneous breast cancer. Tumor reduction was associated with induction of tumor cell apoptosis *in vivo*. Investigation into the potential molecular pathway revealed that treatment with celecoxib caused reduction in activation of anti-apoptotic/pro-survival kinase, Akt. Increased apoptosis was associated with increased expression of pro-apoptotic protein bax, and decreased expression of anti-apoptotic protein bcl-2. Concurrently, we observed decreased tumor cell proliferation and decreased synthesis of VEGF in mammary gland tumors treated with celecoxib *in vivo,* most probably associated with decreased PGE_2 synthesis.

The importance of this study lies in the use of a mouse model system that resembles human disease in many aspects of tumor progression. The MTag tumors start as hyperplasia, like early proliferative lesions seen in the human breast, show indication of histological progression to malignant mammary adenocarcinomas and metastasis, are heterogenous with respect their malignant potential, and trigger signaling pathways inactive in normal breast epithelium (38). One of the pathways that is activated in these mice is the arachidonic acid/COX-2 pathway (52), similar to that described in many human breast cancers. Furthermore, we have recently shown that COX-2 protein as well as its downstream product PGE_2, were highly elevated in human breast tumors and lymph node metastasis compared to normal tissue, with the highest expression being observed in lymph node metastasis (53). There was a direct correlation between increased COX-2 and PGE_2 expression with impaired immune cell function in newly diagnosed stage I and II breast cancer patients (53). Our observations are similar to the reports that have shown

significant elevation of COX-2 protein levels in 43% of human invasive breast cancers and 63% of ductal carcinomas *in situ* (11, 54). Thus, the MTag model offers the potential to evaluate chemoprevention with a highly specific COX-2 inhibitor, celecoxib.

Celecoxib has been shown to target multiple pathways of tumorigenesis including proliferation, apoptosis, angiogenesis, invasion and tumor-induced immune suppression in various breast tumor cell lines. The current report by Chang et al. (51) supports the concept that COX-2 may provide an early target for breast cancer prevention. We demonstrate that early intervention with celecoxib causes reduced primary tumor burden in the Mtag model (Figure 1). We further demonstrate that reduced PGE_2 synthesis (Figures 6A), and reduced PI3K/Akt kinase activation (Figure 4), post celecoxib treatment, may be the mechanism/s underlying enhanced tumor cell apoptosis (Figure 2), and reduced tumor cell proliferation (Figure 5) *in vivo*. Our data are in line with the recent *in vitro* study in prostate cancer cell lines, where it was demonstrated that celecoxib induces apoptosis by blocking or suppressing Akt activation (47). The PI3K/Akt pathway is typically activated in response to oncogenes that bind to receptor kinases at the plasma membrane and lead to the activation of PI3K (55, 56). Activated Akt targets multiple factors involved in cell proliferation, migration and survival/apoptosis. Mechanistically, activated Akt is known to trigger several cyclins including cyclin D1 that affects all stages of the cell cycle, and induces downstream proliferation (56). Preliminary data suggest decreased levels of cyclin D1 in tumor lysates of mice treated with celecoxib, with significant arrest of the mammary tumor cells at the G_2M checkpoint phase of cell cycle (data not shown). Thus far, our results implicate the PI3K-Akt pathway to be critical in the celecoxib-induced apoptosis and inhibition of tumor cell proliferation. However, other pathways such as the Raf/MEK/MAPK

pathway may also be affected by celecoxib and future studies will be designed to evaluate these pathways *in vivo* in the MTag mouse model. One potential mechanism that has been associated with PGE_2-related inhibition of apoptosis is that PGE_2 reduces the basal apoptotic rate by increasing the level of anti-apoptotic proteins such as Bcl-2 (54, 57). Our *in vivo* data support this concept since inhibiting PGE_2 production by targeting COX-2 activity in the MTag tumors led to decrease in Bcl-2 protein levels and concurrent increase in pro-apoptotic protein, Bax (Figure 3A and B) as well as activate effector caspases 3 and 7 (data not shown).

Finally, angiogenesis plays a crucial role in tumor development and progression. COX-2-dependent PGE_2 is a potent inducer of angiogenesis *in vivo* and induces expression of angiogenic regulatory proteins such as VEGF (51, 58, 59). It has been recently shown that overexpression of COX-2 in the mammary gland by MMTV promoter induces mammary carcinogenesis and that the major prostaglandin that is produced in these tumors is PGE_2. These authors further defined the role of COX-2-dependent PGE_2 production in transforming local tumors to invasive cancer by triggering a so called "angiogenic switch" by increasing expression of pro-angiogenic mediators, such as VEGF and its receptors. Thus, we examined whether celecoxib treatment *in vivo* was effective in reducing the exaggerated VEGF levels observed in MTag tumors. Significant decrease in levels of VEGF in the mammary gland tumors was observed (Figure 7), once again suggesting a role of COX-2 and PGE_2 in mediating angiogenesis in the polyoma middle T antigen-induced breast tumors. Although additional mechanisms are involved in mediating the angiogenic effects of COX-2, our data suggest that COX-2 influences angiogenesis at least in part by enhancing VEGF secretion by tumor endothelial cells. Additional studies are needed to fully elucidate the complex events involved in COX-2 mediated

angiogenesis in our model. Our data clearly demonstrate extensive downregulation of PGE_2 in serum (Figure 6A) post celecoxib treatment *in vivo*. PGE_2 binds to cell surface receptors that belong to the family of seven transmembrane domain G protein-coupled receptors, designated EP1, EP2, EP3, and EP4 (54, 60). Future studies will determine the pattern of EP receptor distribution in the MMTV-MTag mice and whether COX-2 inhibitors can modulate EP receptor expression and its activation state.

In summary, celecoxib treatment may exert its antiproliferative, anti-angiogenic, and pro-apoptotic effects by regulating the PGE_2-EP receptor associated pathways, and by decreasing PI3K/Akt phosphorylation. This leads to significant reduction in primary breast tumor burden. Furthermore, this effect may or may not be dependent upon downregulation of COX-2 protein expression in the tumor. Thus, we believe that COX-2 inhibitors not only represents a future therapeutic option for the treatment of human breast cancer in combination with standard therapies, but may also be considered as a potent chemopreventive agent for individuals with high risk of developing breast cancer and for individuals with high risk of disease relapse.

MATERIALS AND METHODS

Generation of MTag mouse model: MTag oncogenic mice was originally a kind gift from Dr. W.J. Muller (McGill University, Toronto, Canada) (36). MTag male mice were mated to C57BL/6 mice to maintain the MTag mice as heterozygous. Approximately, 50% of the pups carry the oncogene and out of that, approximately 50% are females that develop mammary gland adenocarcinomas and are used for the experiments. PCR was used to routinely identify the MTag oncogene. PCR was carried out as previously described (39). Primer pairs for MTag transgene are 5'-AGTCACTGCTACTGCACCCAG-3' (bp 282 – 302) and 5'-CTCTCCTCAGTTCCTCGCTCC-3' (bp 817 – 837). The amplification program for MTag consisted of one cycle of 5 min at 95°C and 40 cycles of 30 sec at 95°C, 1 min at 61°C, and 30 sec at 72°C followed by one cycle of 10 min at 72°C. The PCR product was analyzed by size fractionation through a 1% agarose gel. Amplification of MTag gene results in a 480 bp fragment. All mice are congenic on the C57/BL6 background at $N \geq 10$. All mice were bred and maintained in specific pathogen free conditions in the Mayo Clinic Scottsdale Natalie Schafer Transgenic Animal Facility. All experimental procedures were conducted according to IACUC guidelines.

Celecoxib Treatment: Celecoxib was purchased from Pharmacia Pharmaceuticals as 100mg capsules. Drug was prepared for oral administration as per manufacturer's recommendation. Briefly, the drug was dissolved in dimethyl sulphoxide (DMSO), and rotated at low speed in a 37°C hot room for 12h, centrifuged at 1800 rpm for 10 minutes, supernatant collected, and stored at 4°C as stock solution of 20mg/ml. Ten week old female MTag mice were gavaged orally with 20gauge barrel tip feeding needles (Fine Science Tools, Foster City, CA) at 5, 10 or 20mg/kg

body weight every day (5 days on with 2 days off) for 4 weeks. Control mice were gavaged with DMSO. Six mice per treatment group were used. Following 4 weeks of treatment, mice were sacrificed and mammary tumors dissected and divided into three parts: 1) to generate single cell suspension for flow cytometry, 2) to prepare tumor lysate for western blot analysis and ELISA, and 3) to fix in formalin and embedded in paraffin blocks for immunohistochemical (IHC) analysis. Serum was collected for ELISA. A dose range of 5-20mg/kg body weight was utilized in our spontaneous mouse model based on previous reports in the literature (24, 34). These doses correspond to physiologic dose of celecoxib and are clinically relevant since the doses of COX-2 inhibitors recommended to patients are in the range of 5-20mg/kg body weight (29).

Tumor burden: From 10 weeks of age until sacrifice, control and celecoxib treated mice were palpated once a week for presence of mammary tumors. Palpable tumors were measured by calipers and tumor weight was calculated according to the following formula: g = (L in cm) x (W in cm^2)/2 (39).

Analysis of Apoptosis by Flow Cytometry: Part of the tumor tissue was dissociated to generate single cell suspension by incubating in 5mM EDTA solution for 1h at 37°C. Apoptosis was determined by staining single cells (1 x 10^6) with Annexin V and propidium iodide (PI) using the BD Pharmingen apoptosis kit following the manufacturer's protocol. Cell staining was determined by flow cytometry using the CellQuest program. Percent apoptotic cells were determined by CellQuest statistical analysis program as the cumulative percent cells that were stained positive for both PI and Annexin V (upper right quadrant), and cells that were stained for Annexin V only (lower right quadrant).

Analysis of Apoptosis and proliferation by IHC: Part of the tumor was formalin fixed (10% neutral buffered formalin, pH 6.8-7.2, Fischer Scientific), paraffin embedded and 5μm sections prepared by the Mayo Clinic Scottsdale Histology Core Facility. IHC was performed using the ApopTag Peroxidase *in situ* apoptosis detection kit (Serologicals Corporation, Norcross, GA). 3,3'-Diaminobenzidine was utilized as the chromogen and hematoxylin was used as counterstain. TUNEL positive cells were examined under light microscopy and representative images taken at 200X. For Proliferating Cell Nuclear Antigen (PCNA) staining, paraffin embedded and 5μm sections were subjected to antigen retrieval using the DAKO Target Retrieval (Carpentaria, CA) at 95°C for 40min. Primary antibody (PCNA antibody, BD Biosciences, USA) was used at 5μg/ml at 4°C overnight and DAKO anti-mouse secondary conjugated to horse radish peroxidase (HRP) was used at 1:200 for 2h at R.T. Diaminobenzidine was utilized as the chromogen and hematoxylin was used as counterstain.

ELISA for PGE$_2$ and VEGF: PGE$_2$ and VEGF enzyme immunoassay kits (Cayman Chemical Company, Ann Arbor, MI for PGE$_2$ and Oncogene Research Products, La Jolla, CA for VEGF) were used to assay the levels of PGE$_2$ and VEGF in tumor lysates and serum derived from treated and control mice. All tumor lysates were made in tissue lysis buffer containing 20mM Hepes, 0.15M NaCl, and 1% Triton X-100 supplemented with 80 μl/ml phosphatase inhibitor cocktail II (Sigma P-5726, St. Louis, MO) and 10μl/ml complete protease inhibitor cocktail (Boehringer Mannheim GmbH, Indianapolis, IN). The PGE$_2$ and VEGF assays were performed according to the manufacturer's recommendation. Lysates were diluted appropriately to ensure that readings were within the limits of accurate detection. Results are expressed as picograms

(pg) of PGE_2 or VEGF per milliliter of serum (pg/ml) or per microgram protein of tumor lysate (pg/μg) for individual mice.

Western Blot Analysis for COX-2, phospho Akt, Bax and Bcl$_2$: Tumor lysates from treated and untreated mice prepared as previously stated were resolved by SDS-PAGE on 10-15% resolving gels. 200μg of tumor lysate was loaded per lane. Gels were blotted and probed for COX-2 (p70; 1:200, Santa Cruz, CA), phospho Akt and Akt protein (p60, 1:1000, Cell Signaling, Beverly MA), Bax-HRP conjugated (p23, 1:200, Santa Cruz, CA), and Bcl$_2$ (p26, 1:1000, Trevigen,). Mammary gland tumor lysates from 20-22-week old MTag mouse are used as positive control for COX-2. Jurkat T lymphoma cell lysate was used as positive control for the other proteins. Individual animal protein expression data are shown.

Statistical Analysis: All data are expressed as means $\pm$ Standard Deviation (SD) of the mean. Statistically significant difference between experimental groups was assessed by one-way ANOVA with Dunnett Adjustment.

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FIGURE LEGENDS

Figure 1. Reduced tumor burden in 14-week MTag mice post celecoxib treatment: MTag mice were palpated once a week for presence of mammary tumors. Tumor weights plotted represent total tumor burden (including all mammary glands) per mice at 14 weeks of age (n=9 mice for vehicle, 10mg/kg and 20mg/kg and n=6 mice for 5mg/kg).

Figure 2 A. Increase in Annexin V positive cells in celecoxib-treated MTag tumors *in vivo*: Tumor cells derived from vehicle treated or celecoxib (5, 10, or 20mg/kg body weight) treated MTag mice were stained with Annexin V-conjugated with FITC and PI and percent apoptotic cells (cells positive for Annexin V) analyzed by flow cytometry. Data from six mice per treatment groups are shown. *P* values represent significant difference between celecoxib-treated groups and vehicle control. **B. Increase in TUNEL positive cells in celecoxib-treated MTag tumors *in situ*:** Light microscopic image of TUNEL positive cells visualizing apoptosis *in situ* from mammary gland tumor sections isolated from vehicle-treated and 10mg/kg celecoxib-treated MTag mice. Brown staining represents apoptotic cells. All images are representative of five standardized fields from six separate mice. Images taken at 100X magnification.

Figure 3. Increase in pro-apoptotic protein Bax and decrease in anti-apoptotic protein Bcl-2 post celecoxib treatment: Western blot analysis of Bax and Bcl-2 protein levels in mammary gland tumor lysates, from vehicle and celecoxib (5, 10, 20mg/kg)-treated MTag mice. 100ug of protein loaded per lane. Data from 5 individual mice are shown. Numbers below each lane represents percent of protein expression compared to MTag lysate, which was set to equivalent of 100% as determined by densitometric analysis. Average % expression for each treatment

group (n = 5 mice) is also shown. P values represent significant difference between treatment groups and vehicle control. β-Actin is used as the protein loading control for all tumor lysates.

Figure 4. Decreased phosphorylation of protein kinase B, Akt post celecoxib treatment: Western blot analysis of phospho-Akt and Akt protein levels in mammary gland tumor lysates, from vehicle and celecoxib (5, 10, 20mg/kg)-treated MTag mice. 100μg of protein were loaded per lane. Data from 5 individual mice are shown. Numbers below each lane represents percent of protein expression compared to mouse expressing the most protein, which was set equivalent to 100%, as determined by densitometric analysis. Average % expression for each treatment group (n = 5 mice) is also shown. P values represent significant difference between treatment groups and vehicle control.

Figure 5. Celecoxib induced inhibition of tumor cell proliferation *in vivo* in a dose-dependent manner: Light microscopy images of PCNA staining of mammary tumor sections from vehicle treated (A) and celecoxib (5, 10, 20mg/kg)-treated (B-D) MTag mice. All images are representative of five standardized fields from six separate experiments. Inhibition of proliferation is most evident at 10mg/kg and 20mg/kg dose of celecoxib. Lymph node section (E) and second antibody staining (F) are shown as controls. All images are taken at 200X magnification.

Figure 6. A. Dose-dependent inhibition of PGE_2 synthesis in serum of celecoxib-treated MTag mice: PGE_2 metabolite levels in serum were determined using specific ELISA and values are shown as pg/ml of serum. Serum from mice was collected either before treatment

commenced or after 4 weeks of celecoxib treatment. *P* values represent significant difference between celecoxib-treated and untreated (pre-treatment) or vehicle-treated mice. PGE_2 levels are also compared with serum from age-matched non-tumor bearing wildtype C57BL/6 mice. Significant inhibition is evident at 10mg/kg and 20mg/kg dose. **B. Decrease in COX-2 protein expression is not significant in MTag tumors post celecoxib treatment (n = 5 mice):** Western blot analysis of tumor lysates from untreated, vehicle and celecoxib (5, 10, and 20mg/kg)-treated MTag mice. 100µg of protein was loaded per lane. All tumor lysates expressed COX-2. Untreated and vehicle-treated tumors expressed higher levels of COX-2 than tumor lysates from celecoxib (10mg/kg and 20mg/kg)-treated mice. Numbers below each lane represent percent of protein expression compared to mouse expressing the most protein, which was set equivalent to 100%, as determined by densitometric analysis. Average % expression for each treatment group (n = 5 mice) is also shown. β-Actin is used as the protein loading control for all tumor lysates.

Figure 7. Decreased VEGF levels in MTag tumors from celecoxib-treated mice: VEGF levels were determined in the tumor lysates using specific ELISA and values are shown as pg/µg protein lysate. Tumor lysate was prepared from untreated, vehicle and celecoxib (5, 10, and 20mg/kg)-treated MTag mice. *P* values represent significant difference between celecoxib-treated and vehicle-treated mice. Values are also compared to mammary gland lysate from age-matched non-tumor bearing wildtype C57BL/6 mice. Significant inhibition is evident at 10mg/kg and 20mg/kg dose.
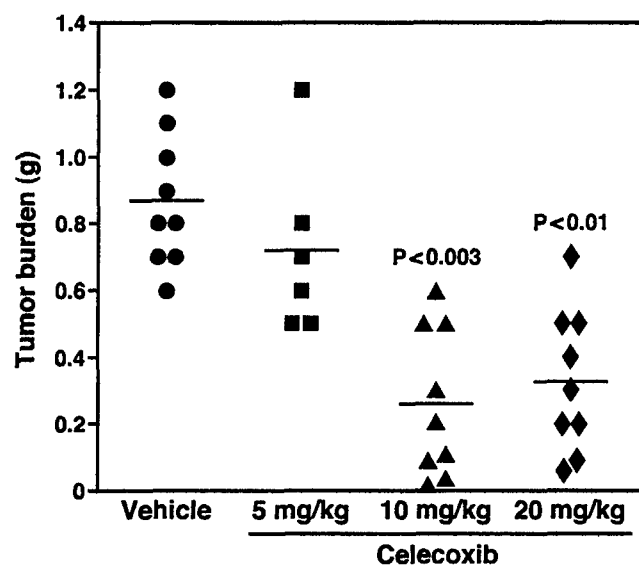
Figure 1
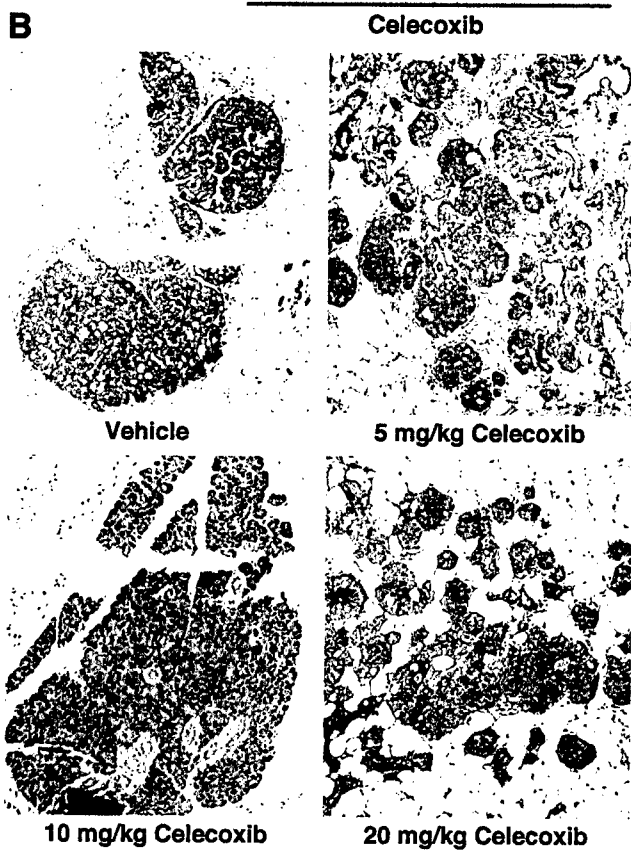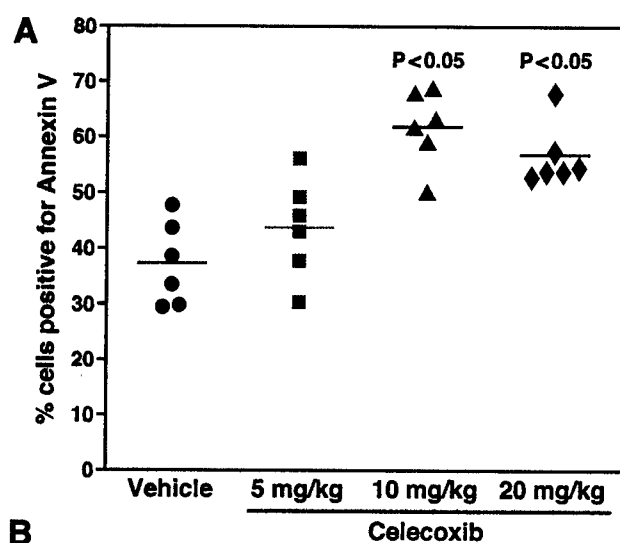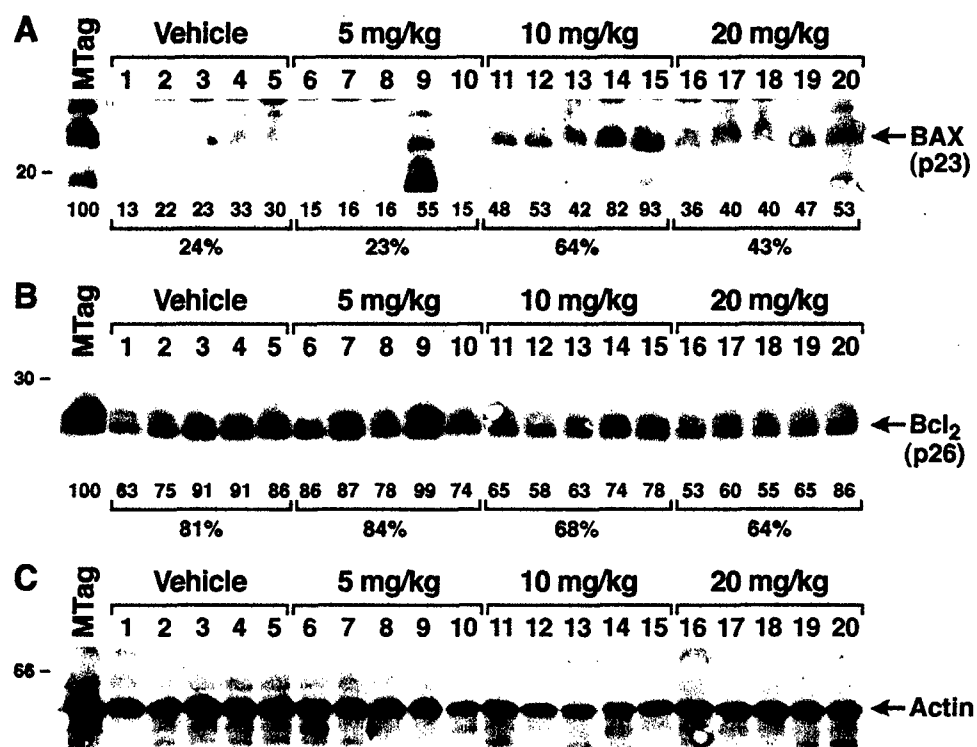
Figure 2

Figure 2
A

% cells positive for Annexin V

Vehicle   5 mg/kg   10 mg/kg   20 mg/kg
                        Celecoxib

P<0.05   P<0.05

B

Vehicle   5 mg/kg Celecoxib

10 mg/kg Celecoxib   20 mg/kg Celecoxib

Figure 3

A

MTag | Vehicle | 5 mg/kg | 10 mg/kg | 20 mg/kg
1 2 3 4 5 | 6 7 8 9 10 | 11 12 13 14 15 | 16 17 18 19 20

20 –

← BAX (p23)

100 | 13 22 23 33 30 | 15 16 16 55 15 | 48 53 42 82 93 | 36 40 40 47 53

24%   23%   64%   43%

B

MTag | Vehicle | 5 mg/kg | 10 mg/kg | 20 mg/kg
1 2 3 4 5 | 6 7 8 9 10 | 11 12 13 14 15 | 16 17 18 19 20

30 –

← Bcl$_2$ (p26)

100 | 63 75 91 91 86 | 86 87 78 99 74 | 65 58 63 74 78 | 53 60 55 65 86

81%   84%   68%   64%

C

MTag | Vehicle | 5 mg/kg | 10 mg/kg | 20 mg/kg
1 2 3 4 5 | 6 7 8 9 10 | 11 12 13 14 15 | 16 17 18 19 20

66 –

← Actin

Figure 4

Phosphorylated Akt

	MTag	Vehicle					5 mg/kg					10 mg/kg					20 mg/kg				
		1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20
pAkt	88	80	62	80	79	100	95	80	90	25	37	81	59	38	64	50	69	76	74	50	51
				80%					65%					58%					64%		

Akt

	MTag	Vehicle					5 mg/kg					10 mg/kg					20 mg/kg				
		1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20
Akt	88	75	75	81	92	100	100	91	98	93	93	92	93	82	87	94	92	95	97	83	90
				90%					95%					90%					91%		

Figure 5

A. Vehicle   B. 5 mg/kg

C. 10 mg/kg   D. 20 mg/kg

E. Lymph node   F. 2° antibody

Figure 6

A

PGE$_2$ levels (pg/ml)

C57/BL6   Pre-   Vehicle   5 mg/kg   10 mg/kg   20 mg/kg
           treatment   Celecoxib

P<0.01 (10 mg/kg)
P<0.01 (20 mg/kg)

B

MTag   Vehicle   5 mg/kg   10 mg/kg   20 mg/kg
        1 2 3 4 5   6 7 8 9 10   11 12 13 14 15   16 17 18 19 20

66 –   ←COX-2
                                                               (p66)

56   51 55 60 67 64   75 61 56 70 40   53 38 40 42 40   44 50 51 46 31

        59%   60%   43%   44%

MTag   Vehicle   5 mg/kg   10 mg/kg   20 mg/kg
        1 2 3 4 5   6 7 8 9 10   11 12 13 14 15   16 17 18 19 20

66 –   ←Actin

Figure 7

Therapeutic efficacy of MUC1-specific cytotoxic T lymphocytes and CD137 co-stimulation in a spontaneous mammary cancer model

Pinku Mukherjee, Teresa Tinder, Gargi D. Basu, Latha Pathangey, Lieping Chen[1], and Sandra J. Gendler
Department of Biochemistry and Molecular Biology and Tumor Biology Program, Mayo Clinic College of Medicine, Mayo Clinic, Scottsdale, AZ 85259
[1]Mayo Clinic, Rochester, MN

Breast cancer immunization strategies were tested in a clinically relevant mouse model of spontaneous mammary gland cancer. MUC1-expressing tumors were elicited by mating MUC1 transgenic mice with MMTV-polyoma middle T antigen (MMTV-PyV-mT) mice, resulting in MMT mice. Response to immunization was determined by following the response to MUC1. MUC1-specific cytotoxic T lymphocytes (CTLs) were adoptively transferred into MMT mice. The cytotoxic T cell clone recognizes an H-2D^b MHC class I-restricted immunodominant epitope of MUC1 (APGSTAPPA). Infused CTLs homed to the tumor bed as detected by flow cytometric analysis. Immunizations of 1x10E7 CTLS every two weeks effectively suppressed tumor growth. However, tumor growth resumed three-four weeks after the last infusion. CTLs recovered from tumors at two weeks after infusion had significantly reduced proliferation and were not cytolytic *in vitro* to MUC1-expressing tumor cells, suggesting they had become tolerized. Tumors in this mouse model mimicked what is often observed in humans, as tumor progression led to down regulation of class I molecules on tumors, secretion of IL-10, active TGF beta, and Cox-2 over expression, which likely elicited the observed tolerance. To overcome tumor-induced tolerance, the CTLs were administered together with co-stimulation provided by CD137 mAb. The CD137 glycoprotein, a member of the tumor necrosis factor receptor family, is expressed on primed CD4$^+$ and CD8$^+$ T cells. CD137 mAb therapy has been suggested to reverse T cell tolerance/anergy induced by tumors. Along with CTL infusion (1x10E7 cells), MMT mice received CD137 mAb weekly (100 ug IP) until sacrifice at 17 weeks. Treatment with MUC1-specific CTLs and CD137 mAb significantly reduced tumor burden (p<0.01). CTLs, sorted from the tumor at sacrifice, were proliferative and cytolytic *in vitro* toward MUC1-expressing tumor cells, suggesting that tolerance had been reversed by CD137 mAb therapy. Current studies are concentrating on strategies to enhance T cell reactivity.

5-30-04

Mayo Clinic Cancer Center

**MUC1/HER-2/neu Peptide Based Immunotherapeutic Vaccines for
Breast Adenocarcinomas**

Principal Investigators/Study Chairs: Svetomir N. Markovic, M.D., Ph.D. *
                                        Sandra J. Gendler, Ph.D.#
                                        Mayo Clinic Cancer Center
                                        200 First Street, SW
                                        Rochester, MN 55905
                                        507/284–2511
                                        507/284-5280 (FAX)

                                        Mayo Clinic Scottsdale
                                        13400 E. Shea Boulevard
                                        Scottsdale, AZ 85255
                                        480/301-7062
                                        480/301-7017 (FAX)

Study Co-chairs: Esteban Celis, M.D., Ph.D.
                   James N. Ingle, M.D
                   Pinku Mukherjee, Ph.D
                   Tom R. Fitch, M.D
                   Barbara A. Pockaj, M.D
                   Edith A. Perez, M.D.

Statistician: Vera J. Suman, Ph.D.

* Investigator having primary responsibility for this protocol
# Study Co-chair not responsible for patient care

Index

Schema

Prior to checking eligibility, contact the Randomization Center (507/284–2753) for study status and dose level.
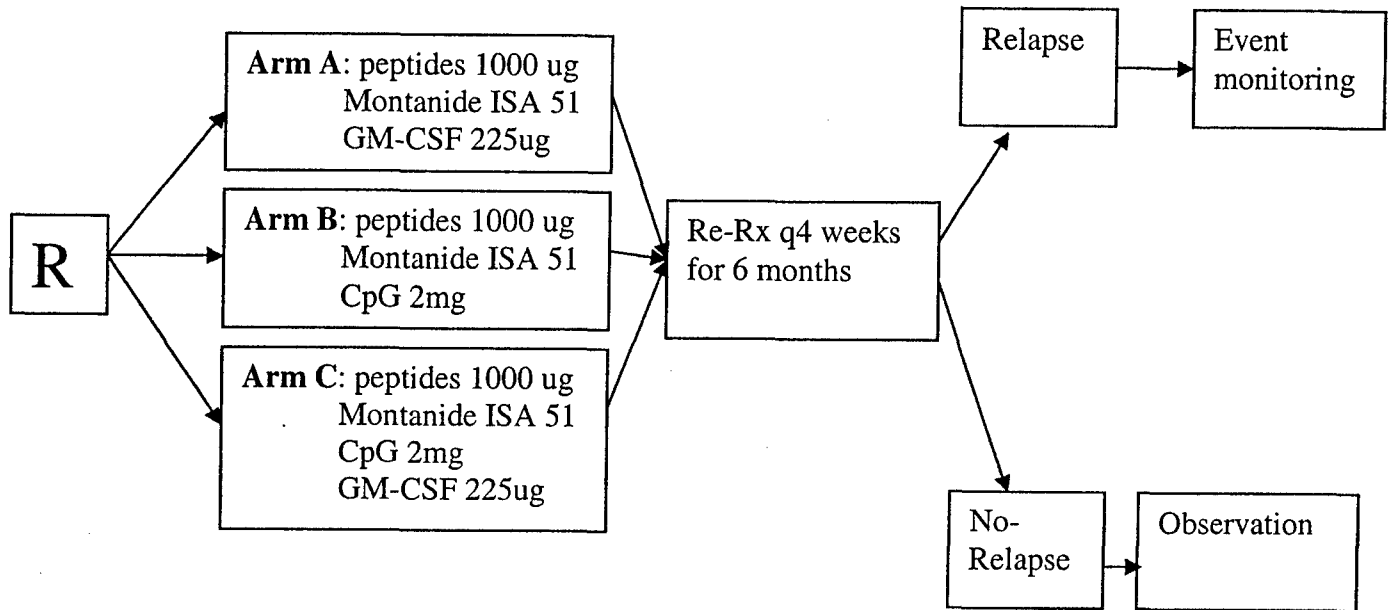
1.0   Background

1.1   Breast cancer is diagnosed in 200,000 individuals in the United States annually and contributes to approximately 40,000 deaths each year. For tumors confined to the breast, surgical removal provides a good prognosis. However, primary tumor that metastasizes to distant sites, such as lymph nodes, lungs, liver and brain, correlates with a poor prognosis. Patients with advanced stage breast cancer are at high risk of relapse. Complications from metastatic disease are the leading causes of cancer-related deaths. Novel adjuvant strategies, such as breast cancer specific vaccines, are being considered as a clinical intervention that may reduce the chance of recurrence.
In recent years there has been great interest in the development of these cancer vaccines, which are designed to immunize individuals to antigens present on tumors. Cancer vaccines are a non-toxic therapy, which have been shown in several melanoma trials to have the potential of controlling disease and prolonging survivalBecause tumors can be surgically removed and there is often a long period of time before the tumor recurs at metastatic sites, cancer vaccines have been proposed as an optimal therapy that could prolong the time to recurrence. This optimal opportunity of immunization in the situation of minimal residual disease has rarely been tested, however, as most vaccines have been given to patients with large tumor burden after the failure of standard therapies in Phase I and Phase II trials. Recently, several gropus have addressed the use of adjuvant immunotherapy following complete surgical resection. Data from these studies is not yet available

1.2   The past two decades in tumor immunology have lead to the discovery of specific tumor antigens that have been shown in preclinical studies to elicit tumor-specific immunity and establish long term memory without autoimmunity. For breast cancer, vaccines composed of epitopes derived of MUC1, HER-2/neu, MAGE3, CEA have been studied and shown to be immunogenic without causing autoimmunity (3-6).

1.3   It is now clear that tumor antigens are presented in the context of specific class I and Class II HLA molecules. Class I presentation, in the presence of appropriate co-stimulation, is thought to stimulate a cytolytic $CD8^+$ T cell response, while antigen presentation in the context of Class II molecules stimulates a $CD4^+$ helper T cell response (7).

1.4   One approach for the development of a cancer vaccine is the use of tumor associated synthetic antigens for immunologic priming. Because specific peptides are ubiquitous in tumors of the same histologic type, identical peptide vaccines may be employed in allogeneic hosts bearing the same tumor histology. Additionally, the use of single peptides for immunization limits the potential induction of undesired autoimmunity (8-10). Recent developments in the use of soluble MHC Class I/peptide tetramers and elispot technology have enabled rapid characterization of epitope-specific CTL responses (11, 12). In addition to being well-explored and understood, many of these antigens are shared tumor antigens. Vaccines that are composed of these antigens can be developed for use in a large number of patients. The primary limitations to peptide based vaccine strategies

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are haplotype restriction, potential for degradation, and uncertainty regarding which peptides, used alone or in combination, are the most immunogenic (13, 14). This study is designed to test these uncertainties.

1.5   One attractive and broadly applicable target for immunotherapeutic strategies is the MUC1 tumor antigen. MUC1, a cell-associated mucin, is expressed on the cell surface of many epithelial malignancies as well as by hematological malignancies (15-18). These include multiple myeloma (92%) and acute myelogenous leukemia (67%) (19). Greater than 90% of breast carcinomas express MUC1; high levels are also found in adenocarcinomas originating from most tissues (15, 17). MUC1 expression is greatly up-regulated on tumors (reviewed in Gendler (20)). Expression on tumors is no longer apical, but it is found all around the cell surface and in the cytoplasm. In addition, glycosylation on tumor-synthesized MUC1 is aberrant, with greater exposure of the peptide core than is found in normal tissues. MUC1 has long been an interesting target molecule for immunotherapeutic strategies, given its high level and ubiquitous expression. Patients with tumors, especially with breast, pancreas and ovarian tumors, have exhibited immune responses to MUC1 with the presence of antibodies and T cells specific for MUC1 detected in about 10% of individuals. An HLA unrestricted T cell response among cancer patients has also been described (21-24). There is increasing evidence from murine and human studies that MHC-restricted T cells can be induced in mice and humans after immunization with the MUC1 peptide or MUC1 antigenic epitopes (25-33). Importantly, there have been reports of two HLA-A2 binding peptides derived from the MUC1 protein (34). One of the peptides is from the tandem repeat sequence of MUC1 and the second peptide is from the signal sequence. MUC1-specific cytotoxic T cells (CTLs) have been induced in T cells from healthy donors following *in vitro* immunization using peptide-pulsed dendritic cells. MUC1-specific CTLs have also been induced *in vivo* after vaccination of breast and ovarian cancer patients with peptide-pulsed DCs (19).

1.6   A second candidate for peptide-based immunotherapy is HER-2/neu, the gene product of the erbB2/neu protooncogene. HER-2/neu is overexpressed in approximately 30% of breast cancer patients. HER-2/neu is also expressed by multiple types of tumors, including ovarian, lung, colon, pancreas and gastric tumors (35-37). HER-2/neu has particular relevance, as it is expressed at high levels in early *in situ* lesions in breast carcinoma (38). Thus, it is a target for early disease. Immunologic responses to HER-2/neu have been detected in a minority of patients with advanced stage breast and ovarian cancer, including antibodies, T helper and CD8 responses (39, 40). Several HLA-class I binding peptides have been previously identified. We recently identified a novel HLA-A2.1 binding peptide from the HER-2/neu extracellular domain [HER-2(9_{435})] (41). This peptide (ILHNGAYSL) bound to HLA-A2.1 with intermediate affinity (IC50 74.6 nM). The HER-2(9_{435}) epitope was tested using an *in vitro* immunization protocol and found to elicit CTLs that killed peptide-sensitized target cells. The CTLs elicited also recognized the HER-2/neu antigens, as it specifically killed tumor cells expressing the HLA-A2.1 and HER-2/neu antigens (see below in

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preliminary data). Furthermore, recognition of the tumor cell targets was significantly inhibited by unlabeled (cold) targets pulsed with HER-2(9_{435}), but not by unlabeled targets either unpulsed or pulsed with a control HLA-A2.1 binding peptide (see below). Thus, the CTLs induced by HER-2(9_{435}) are antigen specific.

A potential limiting factor for peptide based immunotherapy is related to a defined antigenic repertoire which is HLA restricted. This factor, inherent to all peptide-based approaches, restricts patient access. Additionally, because individual peptides only have the potential to induce epitope-specific CTL, the vast majority of potential tumor antigens are not targeted. In this setting, tumor down regulation of individual antigens or HLA epitopes promotes immune evasion. Recent evidence, however, suggests that this problem of epitope restriction may not be as physiologically important as was previously postulated. Specifically, it has now been clearly demonstrated that a T cell response induced against one epitope can stimulate CTL response to other target epitopes through a mechanism termed epitope spreading (4, 42, 43). Using an experimental autoimmune encephalitis model, Vanderlugt et al. have demonstrated that disease progression is associated with the development of epitope-specific helper T cells, which are distinct from those initiating the disease. Transfer of secondary CD4$^+$ cells to naïve mice induces the disease phenotype and the disease is abrogated by blocking the secondary T cell response even though the primary T cell response remains intact (44, 45). Disis demonstrated epitope spreading in 84% of patients vaccinated with HER-2/neu peptides, reflecting the initiation of an endogenous immune response. The immunity persisted after active immunizations ended (4). These data suggest that peptide based approaches to cancer immunotherapy may indirectly stimulate multiple tumor reactive CTL against minor antigens in the presence of residual tumor. Based on this concept, the current study is designed as a therapeutic approach, with peptide epitope selection designed to enhance the number of potential candidates.

In addition to class I epitopes, immunogenic HLA-DR restricted class II epitopes have been defined for HER-2/neu. CD4$^+$ helper T lymphocytes (T$_H$) responses play an essential role in immunologically mediated anti-tumor immunity (46). T$_H$ lymphocytes provide CTLs with growth-stimulating cytokines, prime/activate DCs to effectively present antigen to naive CTL precursors (47-49) and they are important in the development of immune memory (50-52). The development of IgG antibodies to HER-2/neu and the identification of CD4$^+$ T cells that secrete cytokines in response to HER-2/neu peptides or recombinant HER-2/neu protein suggest responses to helper T cells (53-58). We (EC) have identified a promiscuous MHC class II T$_H$ epitope for the HER-2/neu antigen (HER-2$_{883}$). T cell responses are restricted by HLA-DR1, HLA-DR4, HLA-DR52, and HLA-DR53 (59). Peptide-induced T cells were effective in recognizing naturally processed HER-2/neu protein. The peptide HER-2$_{883}$, (KVPIKWMALESILRRRF), which was selected by computer algorithm, was tested for its capacity to stimulate CD4$^+$ T cells isolated from four healthy, MHC-typed individuals (DR1/11, DR1/13, DR4/15, DR7/17) in primary *in vitro* culture

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using peptide pulsed autologous DCs. T cells that proliferated were found to react with peptide and recombinant HER-2/neu intracellular domain protein presented by autologous DCs (see below). These results, showing reactivity with recombinant protein, suggest that HER-2$_{883}$ is naturally processed, as the peptide stimulated T cells react with DCs primed with recombinant protein. Clearly, HER-2$_{883}$ is a naturally processed peptide epitope and is promiscuous for multiple HLA-DR epitopes, making it an ideal candidate for therapeutic applications.

1.7 Because of the expression of MUC1 and HER-2/neu in multiple cancers, the development of this peptide-based immunotherapy can potentially impact the treatment of multiple disease entities, not only adenocarcinomas but hematopoietic malignancies as well. There is considerable interest in the use of the MUC1 peptide vaccination for treatment of multiple myeloma following transplant when there is minimal residual disease prior to remission.

1.9 GM-CSF

Granulocyte-macrophage colony stimulating factor (GM-CSF) is a commercially available cytokine currently used in patients undergoing chemotherapy to shorten the duration of post-chemotherapy neutropenia. Recently published evidence also suggests that GM-CSF may play a role as an immune adjuvant (60). The following observations illustrate the mechanisms by which GM-CSF can potentiate the immunogenicity of an antigen: 1) GM-CSF is a key mediator of dendritic cell (DC) maturation and function (61); 2) GM-CSF increases surface expression of class I and II MHC molecules as well as co-stimulatory molecules of dendritic cells *in vitro* (61); 3) GM-CSF enhances antibody responses to known immunogens *in vivo* (62); 4) tumor cells transfected with genes encoding/expressing GM-CSF are able to induce long lasting, specific anti-tumor immune responses *in vivo* (63); 5) GM-CSF encapsulated in biodegradable microspheres mixed with whole tumor cells resulted in systemic anti-tumor immune responses comparable to those of GM-CSF transfected tumor cells (64). Therefore, addition of GM-CSF to an oligopeptide antigen may substantially enhance its immunogenicity.

In an attempt to optimally enhance the immunogenicity of the peptides we will deliver the antigens and GM-CSF emulsified in incomplete Freund's adjuvant (IFA, Montanide ISA-51). This delivery mechanism should be comparable to a previously demonstrated delivery mechanism utilizing GM-CSF suspended in microspheres and mixed with tumor cells (antigens). We hypothesize that the emulsified GM-CSF in close proximity to tumor antigen peptides will substantially enhance their immunogenicity. This proximity of antigen and GM-CSF seems to be necessary for the adjuvant effect of GM-CSF, as systemic administration of equivalent doses in animal models has not demonstrated adjuvant activity. Also, the adjuvant/local inflammatory properties of IFA may play a role in attracting antigen presenting cells to the site of injection (54, 65). We have preliminary data demonstrating the plausibility of such a mechanism.

1.10 Preliminary data demonstrating the feasibility of this approach already exists. Rosenberg et al. published effective generation of peptide-specific T cells in

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melanoma patients immunized with peptides derived from gp100 (66). Despite the demonstration of a specific immune response, no clinical responses were detected. Addition of systemic GM-CSF resulted in more pronounced CTL and delayed type hypersensitivity reactions and in a few cases objective tumor regressions (64). Salgaller et al. utilized a peptide derived from the gp100 epitope suspended in IFA and demonstrated generation of specific T cell responses to the peptide in melanoma patients (67). Both studies suggest that increased immunogenicity of the peptide antigens leads to a more pronounced T cell response, which in some cases results in a clinically relevant anti-tumor effect. In the proposed study, we will combine the immunoadjuvant effects of both IFA and GM-CSF with the goal of increasing the immunogenicity of the MUC1 and HER-2/neu immunodominant peptides.

Preliminary observations in an ongoing clinical study (MC9973) utilizing HLA-A2 specific melanoma differentiation antigen peptide vaccines in which the peptide is emulsified in a suspension of IFA and GM-CSF is demonstrating enhanced skin reactions if peptide emulsified in IFA is administered in the presence of GM-CSF. A dose of 50 µg of GM-CSF in the presence of IFA and peptide results in extensive local skin reactions as well as evidence of a clinical response in one of seven patients thus far. No changes in the numbers of peptide specific CTLs were observed. However, a recent publication demonstrated superior numbers of vaccine specific CTLs generated in a peptide vaccine utilizing 225ug of GM-CSF in IFA (Slingluff et al, JCO 2003). This would suggest a dose/response relationship of GM-CSF and anti-peptide vaccine CTL frequencies as determined by ELISPOT and tetramer assays. Therefore, in the current trial we propose to use 225ug of GM-CSF suspended in IFA (montanide ISA-51).

1.11   CpG

Therapeutic properties of bacteria in the treatment of malignant diseases (i.e. Coley's toxin) is an observation that has permeated the oncology literature for almost a century. More recently, it has been demonstrated that bacterial DNA possesses unique immunomodulatory features of potential utility in cancer therapy. Specifically, unmethylated CpG are able to stimulate NK cells and B cells. Furthermore, synthetic oligodeoxynucleotide (ODN) constructs containing unmethylated CpG motifs (CpG-ODN) were able to activate dendritic cells (DC) enhancing their antigen processing/presentation properties and stimulating production of Th1 cytokines necessary for CTL immune responses. Thus, CpG ODN appeared to function as an immune adjuvant. Several preclinical and clinical works illustrate the ability of CpG-ODN to function as a potent immune adjuvant for various forms of vaccines. One of the more interesting works, pertinent to this study, demonstrates the ability of CpG ODN to induce CTLs against a peptide vaccine when administered in conjunction with incomplete Freund's adjuvant (IFA) (68). These authors used a MART-1/Melan-A$_{26-35}$ peptide emulsified in IFA with or without the addition of 50ug of CpG ODN to immunize human human D[b] (HHD) A2 transgenic mice. Their data suggest superior anti-peptide immunization in the CpG-ODN immunized group as determined by the frequency

of tetramer positive CTLs. Our own data support these findings demonstrating superior immunization efficacy of IFA+CpG-ODN with ova peptide of C57BL/6 mice when compared to either IFA+peptide or complete Freund's adjuvant (CFA) + peptide (data not shown). An additional benefit to the CpG-ODN adjuvant is that it has been shown to be especially good at enhancing cellular and humoral immunity and promoting a Th1-type of response in older mice (69). The population that develops cancer is mainly older individuals, thus the CpG-ODN adjuvant may be particularly relevant for this trial. Based on preclinical data suggesting the potent immune adjuvant properties of CpG co-emulsified with peptides in IFA, we elected to test the efficacy of CpG-ODN in the setting of a peptide vaccine immunization in this clinical trial. The dose of CpG-ODN that we decided to use in this study is 2mg/vaccine. The dose is based on published data demonstrating a direct dose-dependent relationship of CpG-ODN (0.125 –1.0 mg) and magnitude of measured immune responses (HepB vaccine adjuvant, Halperin SA et al, Vaccine, 2003). This is well below the highest tested doses of 20mg/week. Based on these observations we feel that the 2mg dose is a reasonable starting point for a CpG-ODN adjuvant suspended in Montanide ISA 51 alone or in combination with GM-CSF.

1.12 Preliminary Data

Preliminary data will be presented in multiple sections. First, we will provide data to support the choice of MUC1 and HER-2/erbB2 antigenic epitopes for this trial. Next, we will define our experience using peptides to stimulate tumor reactive T cells for cancer immunotherapy. Finally, we will discuss our experience with the immune adjuvants GM-CS and CpG-ODN. These preliminary data provide a strong foundation for the current proposal.

### 1.12.1 Identification of CTL Epitopes from MUC1

Using a computer analysis of the MUC1 amino acid sequence, two novel peptides were identified with a high binding probability to the HLA-A2 molecule (34). Two peptides from MUC1 were identified; one from the tandem repeat M1.1 ($STAPPVHNV_{950-958}$) and one from the leader sequence M1.2 ($LLLLTVLTV_{12-20}$). The presence of the V in position 6 increases the binding of the M1.1 peptide to the HLA-A2 molecule. There is some variability in the tandem repeats in MUC1 and this sequence is found in the last tandem repeat. Cytotoxic T cells were induced from healthy donors by primary *in vitro* immunization using peptide-pulsed dendritic cells. The peptide-induced CTL lysed tumors endogenously expressing MUC1 in an antigen-specific and HLA-A2-restricted fashion.
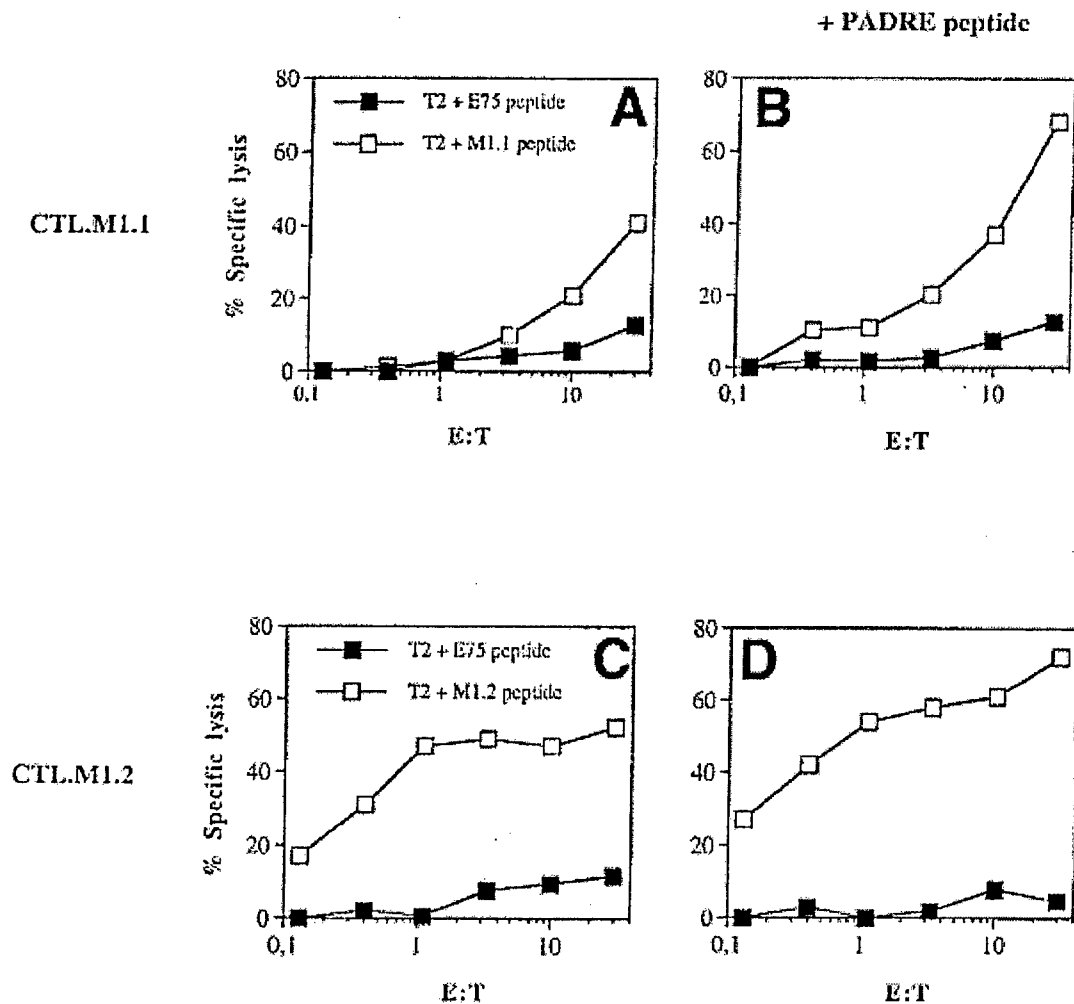
Figure 1. Induction of CTL responses by peptide-pulsed dendritic cells. Adherent peripheral blood mononuclear cells were grown for 7 days with GM-CSF, IL-4, and TNF alpha. DCs pulsed with the synthetic peptides derived from the MUC1 protein (M1.1 and M 1.2) were used to induce a CTL response in vitro. In addition to the MUC1 peptide DCs were incubated with the PAN-DR binding peptide PADRE as a T-helper epitope. Cytotoxic activity of induced CTL was determined in a standard ^{51}Cr-release assay using T2 cells as targets pulsed for 2 hours with 50 μg of the cognate (open symbols) or irrelevant HER-2/neu protein-derived protein derived E75 peptide (solid symbols). (data reproduced from Brossart 1999 (34))

Next, the ability of the induced MUC1-specific CTL lines to lyse tumors expressing MUC1 was tested. MCF-7 cells that express MUC1 endogenously and are HLA-A2 positive were used as targets in a standard ^{51}Cr-release assay. The controls were SK-OV-3 cells, which express MUC1, but are HLA-A2 negative and the immortalized B cell line, Croft, which is A2 positive and was pulsed with MUC1 M1.1 or M1.2 peptides or the irrelevant HER-2/neu E75 peptide.
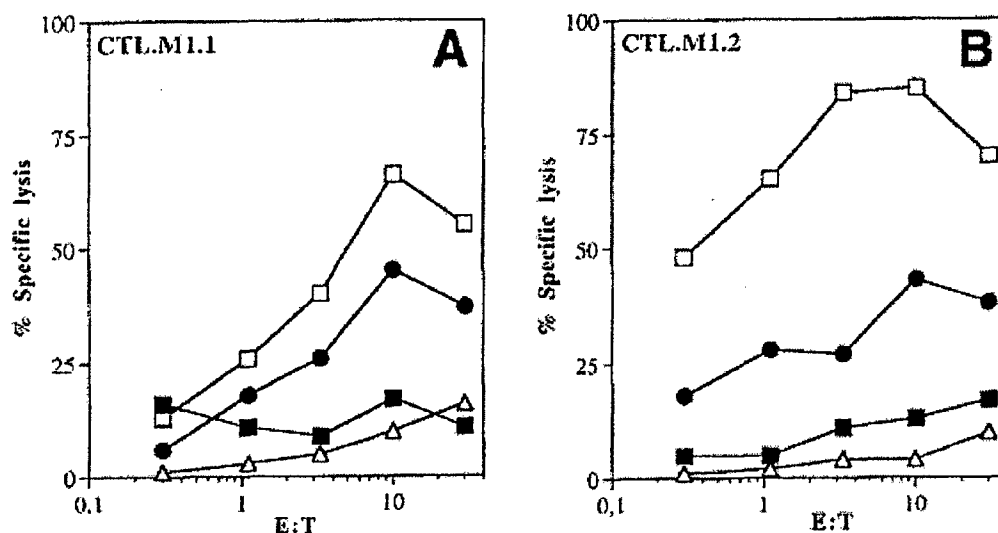
Figure 2. Lysis of cancer cells endogenously expressing MUC1 by CTL.M1.1 (A) and CTL.M1.2 (B). Human breast cancer cell line MCF-7 (HLA-A2$^+$/MUC1$^+$), ovarian cancer cell line SK-OV-3 (HLA-A2$^-$/MUC1$^+$), and the immortalized B-cell line Croft (HLA-A2$^+$/MUC1$^-$) were used as targets in a standard ^{51}Cr-release assay. Croft cells were pulsed with the MUC1 peptides or an irrelevant HER-2/neu-derived peptide E75. (■) Croft + E75 peptide; (□) Croft + M1.1 (A) or M1.2 (B); (●)MCF-7; (Δ) SK-OV-3.

We have chosen to use the M1.1 peptide based on the large amount of data on the response to the MUC1 tandem repeat peptide, both in the human situation as well as in the mouse. Obviously only the human data are relevant for the clinical trials. We will use a HER-2/neu helper epitope (see below, not the PADRE helper epitope)

In the case of HER-2/neu, we have identified a novel CTL epitope HER-2 (9_{435}), which bound HLA-A2.1 with intermediate affinity (IC$_{50}$ 74.6 nM). The peptide identified is: ILHNGAYSL. The .221(A2.1) cell line, produced by transferring the HLA-A2.1 gene into the HLA-A, -B, -C null mutant human lymphoblastoid cell line .221, was used as target (peptide loaded) to measure activity of HLA-A2.1 restricted CTL (70). The CTLs elicited following in vitro stimulation effectively killed HLA-A2.1$^+$ tumor cells, showing that the antigen is appropriately processed by tumors (Fig. 3A). In addition, recognition of the tumor cell target was significantly inhibited by unlabeled (cold) target pulsed with HER-2 (9_{435}) peptide, but not by unlabeled targets either unpulsed or pulsed with a control HLA-A2.1 binding peptide (Fig. 3B).
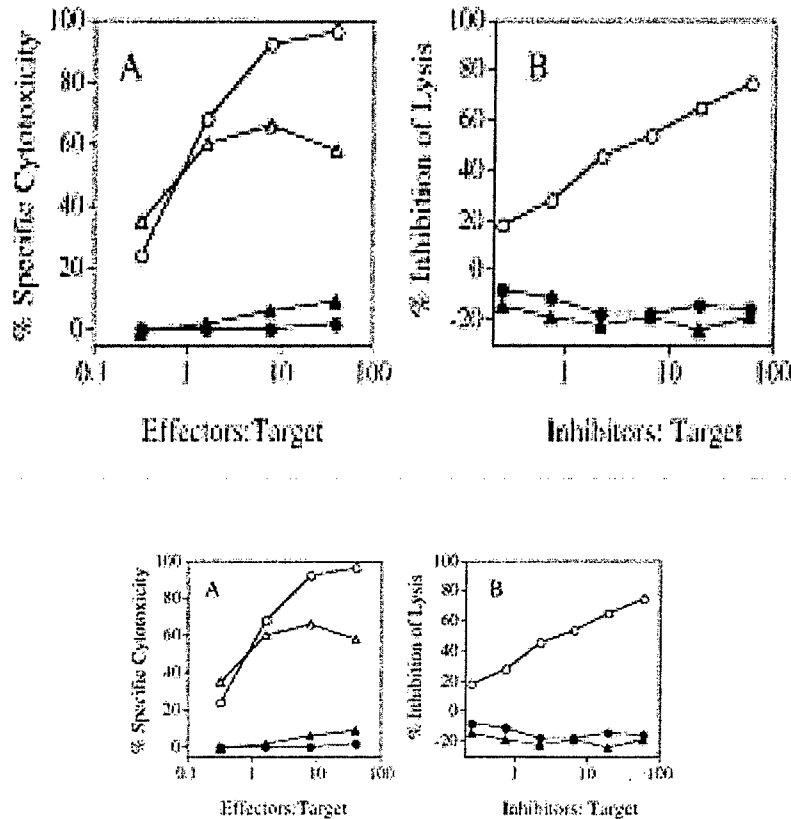
Figure 3. HER-2(9_{435}) specific CTL can kill tumor cells. (A) HER-2(9_{435}) specific CTL were used as effector cells to test for the lysis of the following target cell lines: o, .221A2.1 pulsed with HER-2(9_{435}); ●, .221A2.1 without peptide; Δ, SW403 (colon CA, A2$^+$, HER-2/neu$^+$); ▲, HT-29 (colon ca, A2$^-$, HER-2/neu$^+$). (B): Antigen specificity demonstrated by cold target inhibition assay. Lysis of ^{51}Cr labeled SW403 cells at an effectors/target ratio of 10:1 by the HER-2(9_{435}) specific CTL was blocked at various Inhibitors/Target ratios by the following cold targets: o, .221A2.1 pulsed with HER-2(9_{435}); ▲, .221A2.1 pulsed with irrelevant A2.1 binding peptide (HBc$_{18-27}$); ●, .221A2.1 without peptide.

In addition to the class I epitopes described above, we (EC) have defined a promiscuous MHC class II epitope for HER-2/neu using the algorithm tables published by Southwood et al. (59, 67). The epitope identified is HER-2$_{883}$ (KVPIKWMALESILRRRF). It is important to show that these peptides represent true T cell epitopes that are relevant for the development of tumor immunotherapy. For these experiments autologous PBMCs or DCs were used as APCs and recombinant DNA derived intracellular domain or extracellular domain protein fragments of HER-2/neu were used as a source of antigen. The data in Fig. 4 show that four HER-2$_{883}$-reactive T cell lines proliferated well to HER-2/neu intracellular domain protein, which encompasses the HER-2$_{883}$ peptide but not to HER-2/neu extracellular domain (ECD), which lacks HER-2$_{883}$.
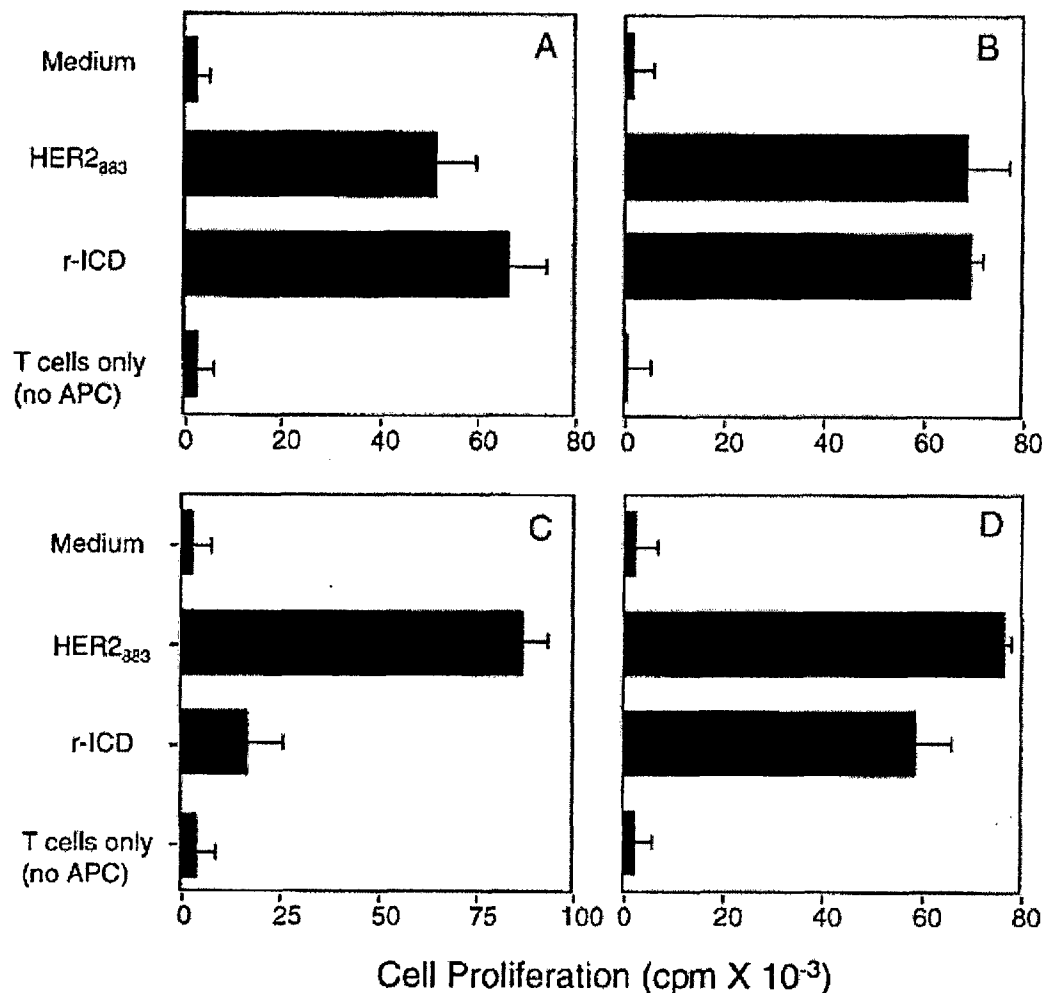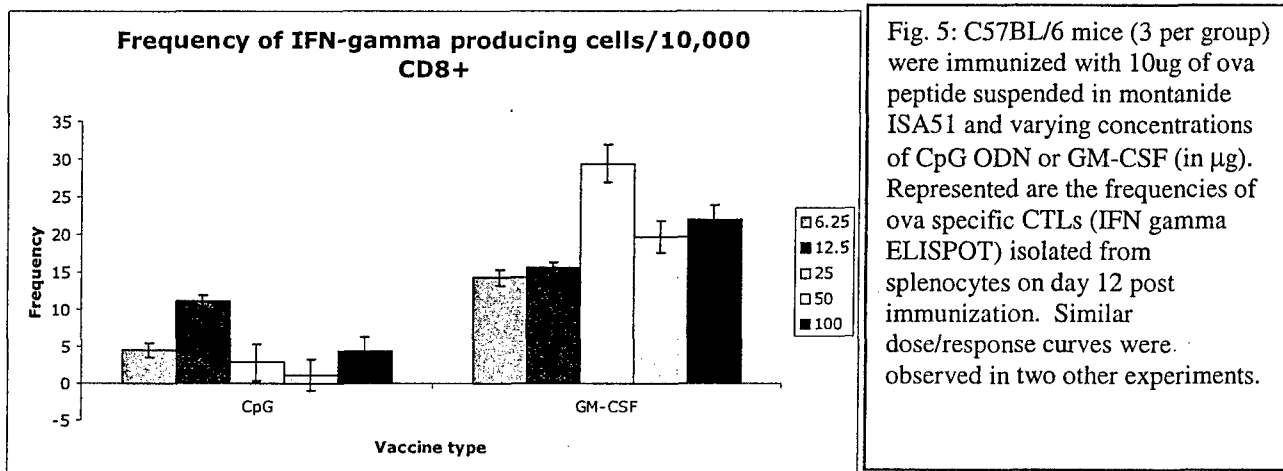
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Cell Proliferation (cpm X 10^{-3})

Figure 4. HER-2_{883}-specific CD4+ T cells can recognize recombinant HER-2/neu intracellular domain (*r-ICD*) protein presented by autologous Dcs in the context of several HLA-DR alleles. The HER-2_{883}-reactive HTLs, TCL-7C (panel *A,* HLA-DR53 restricted), TCL-6D (panel *B,* HLA-DR4-restricted), a clone of TCL-1D (panel *C,* HLA-DR52-restricted), and TCL-1E (panel *D.* HLA-DR53 restricted), were tested for their capacity to proliferate to autologous DCs in the presence of HER-2_{883} peptide (2.5 mg/ml) or recombinant HER-2/neu recombinant ICD protein (10 mg/ml). No significant proliferative response was observed against HER-2/neu ECD protein (data not shown). Values shown are the means of triplicate determinations; *bars,* SD.

1.13 Justification of vaccination strategy

    1.13.1 **Peptide dose** (1000ug): Over the last several years there has been extensive debate over the optimal dose of peptide in a variety of peptide immunization cancer clinical trials. Peptide doses have ranged from 50ug to 2500ug in various studies. The only published study evaluating a peptide vaccine dose-response was performed by Rosenberg et al (69) suggesting that 1000ug of peptide would be reasonable vaccine dose for phase I/II clinical testing.

    1.13.2 **GM-CSF suspended in Montanide ISA 51 as a vaccine adjuvant.** The utility of GM-CSF suspended in montanide ISA 51 as an effective vaccine adjuvant has already been demonstrated in pre-clinical and clinical studies. Our own pre-

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clinical data (Fig 5) demonstrates a bell shaped dose-response curve for GM-CSF co-emulsified with 10ug of *ova* peptide in montanide ISA 51. Two weeks after immunization, the optimal dose of GM-CSF in the mouse model appears to be 100ug. In humans, Slingluff et al demonstrated successful peptide immunization using 225ug of GM-CSF suspended in montanide ISA 51. Up to 80% of treated patietns demonstrated effective immunization with melanoma differentiation antigen peptides. Our clinical data using 10, 50, 75 and 100 ug of GM-CSF suspended with peptides in Montanide ISA 51 failed to demonstrate effective generation of anti-peptide CTLs. In view of these data, we felt that it was reasonable to utilize the same dose of GM-CSF used by Slingluff et al (225ug) with our current set of peptides. If successful, further studies will be performed attempting to generate a dose-response curve of GM-CSF and immunization efficacy similar to that of the mouse model.

Fig. 5: C57BL/6 mice (3 per group) were immunized with 10ug of ova peptide suspended in montanide ISA51 and varying concentrations of CpG ODN or GM-CSF (in μg). Represented are the frequencies of ova specific CTLs (IFN gamma ELISPOT) isolated from splenocytes on day 12 post immunization. Similar dose/response curves were observed in two other experiments.

1.13.3 **CpG suspended in Montanide ISA 51 as vaccine adjuvant.** As described in section 1.11, the co-emulsification of peptide antigens with CpG and Montanide ISA 51 is an effective means of generation of peptide specific CTLs in a pre-clinical model. Our own data confirm these findings using non-transgenic mice immunized with ova peptide co-suspended with CpG in Montanide ISA 51 (Fig. 5). The dose of CpG used in the current study was empirically selected based on the results of a phase I clinical trial utilizing CpG (ISS) as an immune adjuvant for hepatitis B vaccine immunization in healthy volunteers. In this study, volunteers were immunized with an intramuscular injection of hepatitis B vaccine (20ug) mixed with CpG in one of the following concentrations: 225ug, 650ug, 1000ug or 2250ug. A booster injection was administered 2 months later. Serologic data demonstrated (Fig 6) maximal immunization efficacy at CpG doses between 1000 and 2250ug. Based on these data suggesting a bell-shaped dose response curve for CpG (optimum may be between doses 1000ug and 2250ug) as well as our pre-clinical bell-shaped dose response curve, we elected to proceed with a CpG dose of 2000 ug.
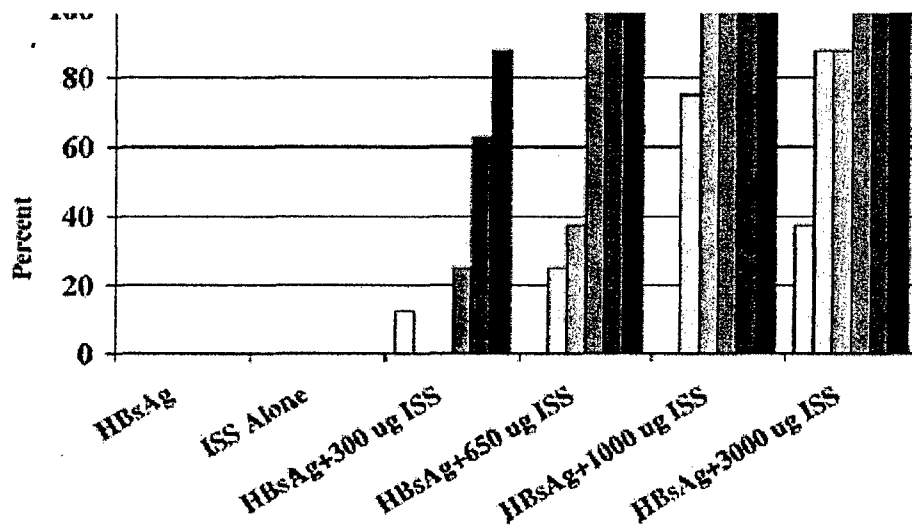
Figure 6: Proportion of participants achieving a protective antibody level (≥10mIU/mL) at various time points after immunization. Time points are (by increasing darkness of bar shade) 7 days after dose 1, 28 days after dose 1, 56 days after dose 1, 7 days after dose 2, 4 months after

2.0 Goals

2.1 To determine the safety and immunization efficacy of MUC1 and HER-2/neu peptide vaccines combined with CpG, GM-CSF or both, as immune adjuvants suspended in montanide ISA-51.

2.2 To describe the impact of immunization on clinical outcomes in patients with MUC1 positive breast cancer.

3.0 Patient Eligibility

Prior to discussing protocol entry with the patient, call the Randomization Center to insure that a place on the protocol is open to the patient.

3.1 Inclusion criteria

3.1.1 Age ≥18 years.

3.1.2 Completed "standard FIRST LINE therapy" (including adjuvant therapy) for breast cancer, clinical stage II and III(at least 12 months prior to registration but no longer than 5 years) and currently with no evidence of disease. Current use of "anti-estrogen" therapy is allowed.

3.1.3 Histologically confirmed adenocarcinoma of the breast treated with surgery, adjuvant chemotherapy, and/or radiation therapy

3.1.4 No radiographic evidence of disease at the time of registration.

3.1.5 MUC1 positive breast cancer by central review.

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3.1.7   HLA-A2 positive.

3.1.8   Laboratory values obtained ≤14 days prior to registration:
- Hemoglobin ≥10.0 g/dL
- Platelets ≥100,000/uL
- ANC ≥1,500/uL
- Creatinine ≤2 ULN
- AST ≤ 3 ULN

3.1.9   Capable of understanding the investigational nature, potential risks and benefits of the study and capable of providing valid informed consent.

3.1.10   Willingness to return to Mayo Clinic Rochester/Scottsdale/Jacksonville for treatment and study-related follow up.

3.1.11   Life expectancy ≥12 weeks.

3.1.12   Willingness to provide the blood and tumor specimens and complete the imaging studies as required by the protocol.
Note: The goals of this study include assessment of the biologic effects on surrogate markers of the agent(s) being tested and are, therefore, contingent upon availability of the blood and tumor specimens and completion of the required imaging studies.

3.2   Exclusion criteria

3.2.1   ECOG performance status (PS) 3 or 4 (see Appendix III).

3.2.2   Uncontrolled infection.

3.2.3   Any of the following:
- Known HIV infection
- Other circumstances (i.e. concurrent use of systemic immunosuppressants and immunocompromising condition) that in the opinion of the physician renders the patient a poor candidate for this trial

3.2.5   Failure to fully recover from acute, reversible effects of prior breast cancer therapy regardless of interval since last treatment.

3.2.6   Any of the following:
- Pregnant women
- Nursing women unwilling to stop breast feeding

- Women of childbearing potential who are unwilling to employ adequate contraception (condoms, diaphragm, birth control pills, injections, intrauterine device [IUD], or abstinence, etc.)

NOTE: This study involves an investigational agent whose genotoxic, mutagenic and teratogenic effects on the developing fetus and newborn are unknown.

3.2.7 Other concurrent chemotherapy, immunotherapy, radiotherapy, or any ancillary therapy considered investigational (utilized for a non-FDA-approved indication and in the context of a research investigation).

3.2.8 Disease-free of prior invasive malignancies for at least 5 years (with the exception of curatively-treated basal cell or squamous cell carcinoma of the skin or carcinoma in situ of the cervix

4.0   Test Schedule

Tests and procedures	≤14 days prior to registration	Prior to each subsequent treatment (q 4 weeks)	Post-therapy acute toxicity evaluation (4 weeks after last Rx)	Long term f/u q3 months for 2 years
History and exam, weight, PS	X	X	X	X
Height	X			
Hematology group: WBC, ANC, Hgb, PLT	X	X	X	X
Chemistry group: total and direct bilirubin, AST, creatinine.	X	X	X	X
HLA class I and II typing[R]	At any time prior to registration			
Serum pregnancy test[1]	X			.
Tumor typing5,[R]	At any time prior to registration			
Tumor evaluation by imaging study (x-ray, CT or PET)[R,2]	X	Every three cycles		X
DTH skin testing (common recall antigens)[3, R]	X	Prior to cycle 6 only		
Research blood specimens	X	X[4]		X[4]

1. Serum pregnancy test obtained ≤7 days prior to registration.
2. Research funding for imaging studies will be provided in cases where f/u imaging is not part of "standard of care".
3. DTH skin testing will be performed using the same complement of antigens in routine use at the treatment site.
4. Research blood samples will be performed before registration as well as prior to **cycles 3, 5 and 6** of therapy as well as every other cycle of long term follow-up **starting at 3 months** after completion of Rx up to two years.
5. Tumor tissues will be stained for MUC1 and HER-2/neu.

5.0   Stratification Factor

Adjuvant chemotherapy: yes versus no
Ongoing anti-estrogen (SERM) therapy: yes vs no
Her-2/neu status: positive vs negative

6.0   Registration/Randomization Procedures

6.1   To register a patient, call (4-2753) or fax (4-0885) a completed eligibility check-list to the Randomization Center between 8 a.m. and 5 p.m. central time Monday through Friday.

6.2 A signed HHS 310 form must be on file in the Randomization Center before an investigator may register any patients.

6.3 Randomization Center personnel will check patient eligibility and the existence of a signed consent form before a patient will be registered into this study.

6.3.1 Patient has given permission to store sample(s) for future research of breast cancer:

6.3.2 Patient has given permission to store sample(s) for future research to learn, prevent, or treat other health problems:

6.3.3 Patient has given permission to give their sample(s) to outside researchers:

6.4 Treatment on this protocol must commence at Mayo Clinic under the supervision of a medical oncologist.

6.5 Treatment cannot begin prior to registration and must begin *3 days after registration.

6.6 Pretreatment tests must be completed within the guidelines specified on the test schedule.

6.7 All baseline symptoms must be documented and graded on the oncology record.

7.0 Protocol Treatment

7.1 As part of the registration process described in Section 6.0, the Mayo Clinic Cancer Center (MCCC) Randomization Center will assign patients to arms 1 through 4

7.2 Treatment Schedules:

Arm A

Arm	Agent		Dose	Route	RxDays	ReRx
A	Montanide ISA-51		1.2mL	subcutaneous injection in un-dissected LN region	Day 1 of Week 1	Q4 weeks (28 days) x 6 cycles
	MUC1 (STAPPVHNV)		1000ug			
	HER-2 peptide-1 (ILHNGAYSL)		1000ug			
	HER-2 peptide-2 (KVPIKWMALESIL RRRF)		1000ug			
	GM-CSF		225ug			

Arm B

Arm	Agent		Dose	Route	RxDays	ReRx
B	Montanide ISA-51		1.2mL	subcutaneous injection in un-dissected LN region	Day 1 of Week 1	Q4 weeks (28 days) x 6 cycles
	MUC1 (STAPPVHNV)		1000ug			
	HER-2 peptide-1 (ILHNGAYSL)		1000ug			
	HER-2 peptide-2 (KVPIKWMALESIL RRRF)		1000ug			
	GpG		2mg			

Arm C

Arm	Agent		Dose	Route	RxDays	ReRx
C	Montanide ISA-51		1.2mL	subcutaneous injection in un-dissected LN region	Day 1 of Week 1	Q4 weeks (28 days) x 6 cycles
	MUC1 (STAPPVHNV)		1000ug			
	HER-2 peptide-1 (ILHNGAYSL)		1000ug			
	HER-2 peptide-2 (KVPIKWMALESIL RRRF)		1000ug			
	GM-CSF		225ug			
	CpG		2mg			

7.3 **Fifteen patients** per Arm (total 45) will be randomly assigned to receive one of the three treatment vaccine preparations. Doses will not be escalated in any individual patient.

Vaccines will be prepared in a single vile and administered as multiple (2-3) subcutaneous injections in regions of un-disturbed axillary or inguinal lymph nodes. Each vaccine cycle will be administered into a single lymph node draining area. Subsequent vaccination cycles will be administered to other (rotating) un-disturbed lymph-node drainage sites. An example of a possible immunization schema of a woman that has undergone left axillary lymph node dissection as part of her primary surgery would be:

Cycle #1: right shoulder
Cycle #2: right thigh
Cycle #3: left thigh
Cycle #4: right shoulder
Cycle #5: right thigh
Cycle #6: left thigh

8.0 Dosage Modification Based on Adverse Events - Adjustments are based on adverse events observed since the prior dose.

ALERT: ADR reporting may be required for some adverse events. See Section 10.

→ → Use Common Toxicity Criteria (CTC) Version 3.0 unless otherwise specified ← ←			
AT SCHEDULED RE-TREATMENT			
CTC CATEGORY	ADVERSE EVENT	AGENT	TREATMENT ADJUSTMENT or OTHER ACTION
ALLERGY/ IMMUNOLOGY	≥Grade 2 allergic reaction/hypersensitivity	Montanide GM-CSF CpG Peptides	Discontinue vaccinations and begin observation.
	≥Grade 2 autoimmune reaction (excluding vitiligo)		Discontinue vaccinations and begin observation.
ALL OTHERS·	≥Grade 3 Hematologic ≥Grade 3 Nonhematologic (excluding alopecia) ≥Grade 2 neurologic	Montanide GM-CSF CpG Peptides	Discontinue vaccinations and begin observation.

9.0 Ancillary Treatment/Supportive Care

9.1 Patients should receive full supportive care while on this study. This includes blood product support, antibiotic treatment and treatment of other newly diagnosed or concurrent medical conditions.

9.2 Patients participating in this clinical trial are not to be considered for enrollment in any other study involving a pharmacologic agent (drugs, biologics, immunotherapy approaches, gene therapy) whether for symptom control or therapeutic intent.

10.0 Adverse Event (AE) Reporting and Monitoring

10.1 This study will utilize the Common Toxicity Criteria (CTC) version 3.0 for adverse event monitoring and reporting. The CTC version 3.0 can be downloaded from the CTEP home page (http://ctep.info.nih.gov/CTC3/ctc_ind_term.htm). All appropriate treatment areas should have access to a copy of the CTC version 3.0.

10.1.1 Adverse event monitoring and reporting is a routine part of every clinical trial. First, identify and grade the severity of the event using the CTC. Next, determine whether the event is expected or unexpected (refer to Section 15.0 and/or product literature) and if the adverse event is related to the medical treatment or procedure (see Section 10.12). With this information, determine whether an adverse event should be reported as an expedited report (see Section 10.2) or as part of the routinely reported clinical data.

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Expedited adverse event reporting requires submission of a written report, but may also involve telephone notifications. Telephone and written reports are to be completed within the timeframes specified in Section 10.2. All expedited adverse event reports should also be submitted to the local Institutional Review Board (IRB).

10.1.2 Assessment of Attribution

When assessing whether an adverse event is related to a medical treatment or procedure, the following attribution categories are utilized:

Definite -   The adverse event *is clearly related* to the investigational agent(s).

Probable -   The adverse event *is likely related* to the investigational agent(s).

Possible -   The adverse event *may be related* to the investigational agent(s).

Unlikely -   The adverse event *is doubtfully related* to the investigational agent(s).

Unrelated -   The adverse event *is clearly NOT related* to the investigational agent(s).

10.2   Expedited Adverse Event Reporting Requirements

Phase I, II and III Studies (Investigational)

	Grade 4 or 5[1] Unexpected with Attribution of Possible, Probable, or Definite	Other Grade 4 or 5 or Any hospitalization during treatment[6]	Secondary AML/MDS[2]
Call Cancer Center Pharmacist[3] within 24 hours	X		
Submit written report within 5 working days[4]	X		
NCI/CTEP Secondary AML/MDS Report Form within 15 working days[5]			X
Submit Grade 4 or 5 Non-AER Reportable Events/Hospitalization Form within 5 working days.[6]		X[6]	

1. Includes all deaths within 30 days of the last dose of investigational agent regardless of attribution or any death attributed to the agent(s) (possible, probable, or definite) regardless of timeframe.
2. Reporting for this AE required during or after treatment.

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3. Contact the Cancer Center Pharmacist (Mayo Clinic - Rochester) at (507) 284-2701.
4. Use *Adverse Event Expedited Report – Single Agent* or *Multiple Agents* report form. Submit to the Cancer Center Pharmacist (Mayo Clinic - Rochester) and to the Cancer Center Protocol Development Coordinator (PDC) for IRB reporting. The pharmacist will report to the Food and Drug Administration (FDA) as required.
5. Submit per form-specified instructions and provide copy to Cancer Center Pharmacist and Cancer Center PDC. The PDC will facilitate IRB as warranted by the event.
6. In addition to standard reporting mechanism for this type of event, submit information to the PDC. The PDC will facilitate IRB reporting as warranted by the event. If Adverse Event Expedited Report – Single Agent or Multiple Agents report form was completed, this form does not need to be completed.

10.3 Adverse events to be graded at each evaluation and pretreatment symptoms/conditions to be evaluated at baseline per Common Toxicity Criteria (CTC) Version 3.0 grading unless otherwise stated in the table below:

CTC Category	Adverse event/Symptoms	Baseline	Each evaluation
Constitutional Symptoms	Fatigue	X	X
Dermatology/Skin	Injection site reaction		X
	Rash/desquamation	X	X
Pain	Arthralgia	X	X
	Myalgia	X	X
	Bone pain	X	X

10.3.1 Documentation of the following AEs, when experienced by a participant but not specified in Section 10.3, are to be submitted using the case report forms:

10.3.1.1 Grade 1 and 2 AEs deemed possibly, probably, or definitely related to the study treatment or procedure.

10.3.1.2 Grade 3, 4, or 5 AEs and deaths within 30 days of the participant's last treatment, regardless of attribution to the study treatment or procedure, with the exception of signs or symptoms definitely related to the participant's disease or disease progression.

10.3.1.3 Any death more than 30 days after the participant's last study treatment or procedure that is felt to be at least possibly treatment related must also be submitted as a Grade 5 AE, with a CTC type and attribution assigned.

11.0 Treatment Evaluation

11.1 For the purposes of this study, patients should be re-evaluated every 12 weeks.

11.2   At the time of reevaluation, patients will be classified in the following manner:

11.2.1 No evidence of disease (NED).

11.2.2 Breast cancer recurrence (REC). Local/regional breast cancer recurrence is defined as the development of tumor (except LCIS) in the ipsilateral breast (after lumpectomy); in the soft tissue/chest wall and/or skin of the ipsilateral chest wall; or tumor in the ipsilateral internal mammary, infraclavicular, or axillary nodes or soft tissue of ipsilateral axilla. Suspected tumor recurrence in the ipsilateral breast, chest wall structures or lower (level I ± II) axillary nodal areas must be confirmed by biopsy or cytology. Histologic or cytologic confirmation of tumor is recommended for internal mammary or infraclavicular/high axillary nodal recurrence. A distant recurrence is defined as development of tumor in areas other than the local/regional area that is documented by a positive cytology aspirate, biopsy, or imaging studies.

11.2.3 New primary (NEWP): A new primary is defined as the development of contralateral breast cancer or a second cancer other than squamous or basal cell carcinoma of the skin, carcinoma in situ of the cervix or LCIS of the breast that is histologically confirmed.

11.3   Further treatment after the documentation of a breast cancer recurrence or second primary cancer is left to the discretion of the treating physician.

12.0   Descriptive Factors - None

13.0   Treatment/Follow–up Decision at Evaluation of Patient

13.1   Patients who have not recurred at time of their reassessment and have not experienced intolerable toxicity may continue protocol treatment at the same dose level for a maximum of 6 cycles or until progression of disease, a second primary or an intolerable adverse event occurs.

13.2   Patients who develop progression of disease, a second primary or intolerable toxicity will be removed from protocol treatment and go to the event monitoring phase of the study. Subsequent treatment is at the discretion of the treating physician.

13.3   Patients may refuse further protocol treatment at any time and go to the event-monitoring phase of the study.

13.4   If a patient is declared ineligible by the study team, on-study material, treatment evaluation forms, and end of treatment form should be submitted. No further follow-up after notification of ineligibility is required.

13.5 If a patient is declared a cancel by the study team, on-study material should be submitted. No further follow-up is required.

14.0 Correlative/Translational Studies

14.1 Description of Assays

Active vaccines for the immunotherapy of solid tumors have met with only limited success. It is our hypothesis that the causes of this failure are multifactorial and can be improved by the inclusion of stringent patient selection criteria, careful dose titration based on immunologic response monitoring, and correlation of immunologically based dosing parameters with clinical outcome. The following sections define the strategies that will be employed in this trial to evaluate immunologic response to MUC1, and HER-2 peptides.

14.1.1 Immune Responses to T Helper and CTL Epitopes

14.1.1.1 Elispot

Estimates of frequencies of peptide-specific, IFNγ- and IL-5-producing cytotoxic T lymphocytes and helper T lymphocytes will be obtained by ELISPOT assays following *in vitro* stimulation with peptide-sensitized stimulator cells [87, 89]. IL-5 production, rather than that of IL-4, will be assayed because of the increased signal:noise ratio [89]. CD8+ and CD4+ T cells will be positively selected by magnetic activated cell sorting (MACS, Miltenyi Biotech) from cryopreserved and thawed peripheral blood lymphocyte buffy coat. Antigen-presenting cells (APCs) will also be isolated from CD4-/CD8-cell population by MACS (beads and reagents purchased from Miltenyi Biotech). CD8+ and CD4+ responder T cells will be stimulated with irradiated APCs pulsed with the target peptides used for vaccination. After 5 days of co-culture, the responding cells will be diluted, titrated, and re-stimulated with APCs pulsed with target peptides for 24 hours in 96 well microtiter ELISPOT plates coated with IFNγ- or IL-5-specific capture antibody (ELISPOT Kit purchased from MABTECH, Stockholm, Sweden). The target peptides for re-stimulation include the peptide used for primary stimulation (MUC1 and HER-2 peptides) and a negative control peptide (YIGEVLVSV). The wells are washed and treated with ALP-conjugated secondary antibody and cytokine-producing spots detected using appropriate substrate (all reagents are provided in the kit). After stopping the reaction, the developed microtiter plates are shipped to Zellnet Consulting in New York for evaluation of number of spot-producing cells for each responder cell titration. All analyses are performed by the

consulting firm and data provided electronically to the investigator. The difference between the frequency of spot-producing cells obtained with the target peptides and control peptide will determine the frequency of peptide-specific, cytokine-producing CD4+ or CD8+ T cells.

14.1.1.2   Tetramers

The estimation of frequencies of CTLs that recognize specific peptides bound to class I molecules became increasingly easier and more quantifiable with the construction and application of class I tetramers (12). Tetramers are comprised of four MHC class I molecules designed to express a single 9 mer peptide epitope of interest and are conjugated to biotin. We will use MUC1 (**STAPPVHNV**) and HER-2/neu peptide (**ILHNGAYSL**) epitopes to create a panel of MHC-class I tetramers. This will enable us to detect T cell responses against MUC1 and CEA in the periphery pre and post immunization. On the day of staining, test PBLs are thawed, washed, and resuspended in the staining buffer at 10×10^6 cells/ml. Peptide-specific tetramers (1ug/ml) are added to 100ul cell volumes and incubated for 30 min at 4°C. This cell suspension is then stained with streptavidin-PE for 30min on ice, washed and stained with FITC-anti-CD8, and APC-anti-CD3 antibodies for 15 min at room temperature. The stained cells are analyzed with FACSCAN instrumentation and CellQuest software (BD Biosciences); a minimum of 5×10^5 cells/sample is analyzed for accurate estimation of CTLs with low frequencies. The analysis involves (1) gating on lymphocytes using forward and side-scatter; (2) gating on allophycocyanin-positive PBLs that stain with anti-CD3, and (3) analyzing the gated cells for PE and FITC staining. The frequency of doubly stained cells (trimer+/CD8+) will be estimated for each of three replicate wells for calculation of the mean frequency (±s.e). All assays will include a negative control tetramer that includes the HLA-A*02-binding YIGEVLVSV peptide (72).

14.1.2 Antigenic Profiling

14.1.2.1   Expression of Class I HLA Antigens on tumor tissue.

Initial entry criteria require HLA-A typing of peripheral blood with subsequent confirmation of HLA class I antigen expression on tumor cells by immunohistochemistry. One of the mechanisms by which tumors are postulated to evade the

immune response is by down regulation of classical HLA molecules necessary for antigen presentation.

14.1.2.2 Tumor Expression of MUC1 and HER-2

Tumor blocks will be used to determine the levels of expression of HER-2 and MUC1 on breast cancer tumor cells obtained at the time of most recent surgical resection. HER-2 expression will be determined using a clinical grade test +1 to +3 and levels of expression will be graded on a semi-quantitative scale. MUC1 expression will be determined by positive staining with one of three antibodies to MUC1 (HMFG-2, BC-2, or B27.29). Negative controls will be incubated with PBS instead of monoclonal antibody. Staining of cytoplasm and plasma membrane will be evaluated. Cells will be considered positive when at least one of these components is stained. Antibody staining patterns will be scored in a semi quantitative manner from +1 to +3.

14.1.3 Sample Schedule

14.1.3.1 Blood

100 mL of blood will be collected (heparin) prior to registration and prior to each subsequent immunization as well as every 3 months after conclusion of active therapy until 24 months following registration.

14.1.3.1 Tumor

Tumor blocks will be collected from the patient's most recent surgery prior to study registration.

14.1.4 Sample Preparation

14.1.4.1 Blood

Peripheral blood lymphocytes (PBLs) are enriched by flotation over Ficoll-Hypaque and frozen in aliquots in 10% DMSO for storage at -150°C. Percentages of CD4+ and CD8+ T cells, B cells, monocytes, and dendritic cells are estimated by flow cytometry with a panel of specific monoclonal antibodies. In addition, proliferation assays (3H-thymidine uptake) are performed to estimate T cell responses to polyclonal stimulus (phytohemagglutinin), target antigens (MUC1 and HER-2/neu) and a recall antigen (tetanus toxoid). These two sets of experiments are important for estimating the representation of

individual lymphoid populations and evaluating overall T cell responsiveness. CD8+ (CTLs) and CD4+ (HTLs) are positively purified from cryopreserved and thawed PBLs by magnetic bead separation (Miltenyi Biotek). Additionally, serum will be collected and stored from each of these samples. Cells will then be frozen and stored at -150° for future use.

14.3   Delayed-type hypersensitivity (DTH) skin testing

Skin testing (baseline - prior to registration) will be coordinated through the Mayo Immunization/Allergy Clinic (L-15). A typical panel includes candida, mumps, PPD, and trichophyton. Other antigens may be substituted in the event of antigen unavailability. Patients will return for 1-2 follow-up measurements consistent with L-15 procedures. Patients must have a "positive" reaction to at least one of the antigens tested, to be considered eligible for participation. Patients with only "doubtful" or "negative" reactions will not be considered eligible.

15.0   Drug Information

15.1   **MUC1 (STAPPVHNV)-** *Investigational supply*

15.1.1   Other Names: epithelial membrane antigen (EMA), polymorphic epithelial antigen (PEM), DF3 antigen, Ca1, MAM-6, H23, episialin

15.1.2   Formulation and Storage: to be determined
Samples will be vialed (glass vials with teflon coated stoppers) as powder at a concentration of 1.2mg/vial and kept frozen at –20°C until use.

15.1.3   Drug Procurement and Accountability: to be purchased from Clinalfa

15.2   **HER-2 Peptide-1 (ILHNGAYSL)** - *Investigational supply*

15.2.1   Other Names: erbB2, neu

15.2.2   Formulation and Storage: to be determined
Samples will be vialed (glass vials with teflon coated stoppers) as powder at a concentration of 1.2mg/vial and kept frozen at –20°C until use.

15.2.3   Drug Procurement and Accountability: to be purchased from Clinalfa

15.3   **HER-2 Peptide-2 (KVPIKWMALESILRRRF)** - *Investigational supply*

15.2.1   Other Names: erbB2, neu

15.2.2   Formulation and Storage: to be determined

Samples will be vialed (glass vials with teflon coated stoppers) as powder at a concentration of 1.2mg/vial and kept frozen at –20°C until use.

15.2.3 Drug Procurement and Accountability: to be purchased from Clinalfa

15.4 Montanide ISA-51 Adjuvant [MONTAN] - *Investigational supply*

15.4.1 Formulation and Storage

Montanide ISA-51 is an oil-based adjuvant product similar to Incomplete Freund's Adjuvant. Which when mixed with a water-based solution on 1:1 w/w ration, forms a water-in-oil emulsion. It consists of highly purified oil, Drakol VR, and a surfactant, mannide oleate. Montanide ISA-51 is manufactured by Sepic, Inc., and is provided in amber glass ampoules containing 3 mL of the solution. Montanide ISA-51 will be purchased from Seppic Inc.

15.4.2 Mode of Action

Acts to enhance immune response to vaccination; the precise mode of action is unknown.

15.4.3 Storage and Stability

The solution is stored at controlled room temperature. Exposure to cold temperatures may result in a clouded solution, which should be discarded. An expiration date is printed on the ampoule label.

15.4.4 Compatibilities/Incompatibilities

The oil may break down the rubber tip of the plunger on syringes; it is advisable to use a different syringe for each ampoule. Do not allow the Montanide ISA-51 to be in direct contact with the rubber tip of the plunger for more time than is necessary to withdraw the solution and inject it into the peptide vial. Fresh syringes will be needed to withdraw the emulsified vaccine from the vaccine vial. Once the emulsion is made, there is less interaction of the oil directly with the rubber tip of the plunger.

15.4.5 Drug Procurement and Accountability

Montanide ISA-51 will be purchased from Seppic Inc. The Cancer Center Pharmacy Shared Resource will store the drug and maintain records of inventory and disposition of all agent received.

15.4 GM-CSF (sargramostim, Leukine®)

15.4.1 Preparation and Storage

Liquid (used in this study) is available in vials containing 500 mcg/mL (2.8×10^6 IU/mL) sargramostim. Carton of 5 multiple-dose vials; each vial contains 1 mL of preserved 500 mcg/mL LEUKINE Liquid (NDC 50419-050-30). LEUKINE should be refrigerated at 2-8°C (36-46°F). Do not freeze or shake. Do not use beyond the expiration date printed on the vial.

15.4.2 Known Potential Toxicities

Fever, chills, asthenia, malaise, rash, peripheral edema, dyspnea, headache, pericardial effusion, bone pain, arthralgia, and myalgia.

15.4.3 Drug Procurement:

Leukine 500 mcg vials are available commercially. Drug will be purchased using study grant funds (i.e. Patients will not be charged for GM-CSF).

15.5 CpG-7909 (ProMune™)

15.5.1 Preparation and Storage
CpG-7909 is formulated as a sterile phosphate buffered saline solution (5mg/mL) stabile for parenteral administration. The sterile and pyrogen free solution contains no preservatives. Vials are intended for single entry: penetration of the vial's stopper should only be done once to maintain sterility. The drug product is packaged in clear, Type I USP glass vials with teflon-coated stoper closure and flip-caps. The drug product should be stored under refrigeration (2-8oC). CpG-7909 is stable for at least one year if stored frozen.

15.5.2 Known potential toxicities:
The list of reported serious adverse events with the use of CpG-7909 demonstrates the following toxicities:
1. **Related**: reactive follicular lymphatic hyperplasia.
2. **Unlikely Related**: anemia, superior vena cava syndrome, dyspnea, malignant ascites, post-operative bleeding, hepatic failure, renal failure, post-operative wound infection, GI hemorrhage, prolonged coagulation time, bacteriemia, ureteric obstruction, congestive heart failure, DVT, vomiting, dehydration, vein compression, hydronephrosis, urinary retention, proctalgia, hypercalcemia, pleural effusion, subacute inflammatory demyelinating polyneuropathy, pelvic inflammatory disease, unstable angina, myocardial infarction, atrial fibrilation and grand mall seizures.

15.5.3 Drug Procurement: to be purchased from Coley Pharmaceutical Group Inc.

15.6 Vaccine Preparation Instructions

15.6.1 General Vaccine Preparation Information

Emulsify the peptides/GM-CSF or CpG mixture with Montanide ISA-51. Prepare the vials as directed for each group below. Place the vial upside down in a tube platform holder of a vortex machine and vortex at highest speed for 12 minutes. This will minimize the amount of emulsion adhering to the inside surface of the vial. Because neither the peptide solution nor the Montanide ISA-51 contains preservatives or bacteriostatics, the prepared peptide vaccines should be administered as soon as possible.

15.6.1.1 Arm A

Remove the peptide vials from the freezer and thaw at room temperature. Remove a vial of liquid GM-CSF (500 mcg/mL) from the refrigerator and allow to reach room temperature. Withdraw 700µL of GM-CSF and add to the peptide vial containin 1.2 mg of peptide. Suspend the first peptide in the solution, withdraw the mixture and add it to the 2nd peptide vial. Repeat the same procedure and re-suspend the 3rd peptide vial. In the 3rd vial also 1.0 mL of Montanide ISA-51 to the peptide vial. Place the vial upside down in a tube platform holder of a vortex machine and vortex at the highest speed for 12 minutes. This will minimize the amount of emulsion adhering to the inside surface of the vial. Load two tuberculin syringes with equal volumes of this emulsion prior to use). Correct emulsification will be tested by carefully placing a small droplet of the emulsion on the surface of ice-cold distilled water (in a small 10 mL beaker) and observing that the droplet does not disperse after 2 minutes. Discard unused GM-CSF and peptide solution. Each syringe will be identified with the patient's name and confirmed by a second pharmacist. The nurse will administer the vaccine mixture to the patient as soon as possible.

Arm B

Remove the peptide vials from the freezer and thaw at room temperature. Remove a vial of liquid CpG-7909 (5mg/vial/mL) from the refrigerator and allow to reach room temperature. Withdraw 0.5mL of CpG-7909 and add to the first peptide vial containing 1.2 mg of peptide. Suspend the first peptide in the solution, withdraw the mixture and add it to the 2nd peptide

vial. Repeat the same procedure and re-suspend the 3rd peptide vial. In the 3rd vial also add 1.0 mL of Montanide ISA-51. Place the vial upside down in a tube platform holder of a vortex machine and vortex at the highest speed for 12 minutes. This will minimize the amount of emulsion adhering to the inside surface of the vial. Load two tuberculin syringes with equal volumes of this emulsion prior to use). Correct emulsification will be tested by carefully placing a small droplet of the emulsion on the surface of ice-cold distilled water (in a small 10 mL beaker) and observing that the droplet does not disperse after 2 minutes. Discard unused CpG and peptide solution. Each syringe will be identified with the patient's name and confirmed by a second pharmacist. The nurse will administer the vaccine mixture to the patient as soon as possible.

Arm C

Remove the peptide vials from the freezer and thaw at room temperature. Remove a vial of liquid GM-CSF (500 mcg/mL) from the refrigerator and allow to reach room temperature. Remove a vial of liquid CpG-7909 (5mg/vial/mL) from the refrigerator and allow to reach room temperature. Withdraw 0.5mL of CpG-7909 and add to the first peptide vial containing 1.2 mg of peptide. Suspend the first peptide in the solution, withdraw the mixture and add it to the 2nd peptide vial. Repeat the same procedure and re-suspend the 3rd peptide vial. In the 3rd vial also add 1.0 mL of Montanide ISA-51 to the peptide vial. Finally, add 0.6mL of GM-CSF to the mixture. Place the vial upside down in a tube platform holder of a vortex machine and vortex at the highest speed for 12 minutes. This will minimize the amount of emulsion adhering to the inside surface of the vial. Load two to three tuberculin syringes with equal volumes of this emulsion prior to use). Correct emulsification will be tested by carefully placing a small droplet of the emulsion on the surface of ice-cold distilled water (in a small 10 mL beaker) and observing that the droplet does not disperse after 2 minutes. Discard unused GM-CSF, CpG-7909 and peptide solution. Each syringe will be identified with the patient's name and confirmed by a second pharmacist. The nurse will administer the vaccine mixture to the patient as soon as possible.

15.7   Vaccine Administration Information

15.7.1 Dose Specifics

Each peptide vaccine will consist of a total volume of approximately 2 to 3 mL, containing the correct dose of the peptide(s), Montanide ISA-51, and GM-CSF or CpG. Be sure to confirm the proper cohort and dose level before preparing the product. Refer to section 7.11, 7.12, and 7.13 for correct dosing level within the proper cohort.

15.7.2 Administration

Vaccinations will be given on day 1 of each treatment cycle. Due to the large volume, each peptide vaccine is administered in 2 to 3 shots in a contiguous location the peptide vaccine should be injected in the vicinity of one of the major nodal basins. This basin must not have been dissected.

15.8 Vaccine Side Effects:

15.8.1 Because of the low dose of GM-CSF used and the slow release nature of the vaccine emulsion, side effects normally seen with systemic treatment doses of GM-CSF should not play a factor in this vaccination treatment. Expected side effects are related to the peptides and Montanide ISA-51. It is possible that the GM-CSF and CpG-7909 may potentiate the reaction seen at the injection site.

15.8.2 Dermatology/Skin: Injection site reaction, rare granuloma formation, possible development or worsening of pre-existing vitiligo, rash.

15.8.3 Hepatic: transient rises in liver transaminases.

15.8.4 Constitutional: Low-grade fever.

16.0 Statistical Considerations and Methodology

16.1 Study design: This is a pilot study designed to determine the immunologic effects of a MUC1/HER-2/neu peptide based tumor vaccine in the presence of the immune adjuvant GM-CSF suspended in montanide ISA-51, CpG suspended in montanide ISA-51 or the combination of GM-CSF and CpG co-emulsified in montanide ISA 51.

16.1.1 A secondary goal of the study is to determine the toxicity profile of each treatment combination

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16.2 The study design chosen for this proposal is a stratified randomized design with type of dominant disease (see section 5.0) as the sole stratification factor. Toxicities will be carefully monitored and accrual will be suspended if 2 or more of the first six patients experience a grade 4 hematologic toxicity lasting for 5 or more days. In the event of at least two patients experiencing immunologic toxicity $\geq$ grade 2 or any toxicity $\geq$ grade 3 accrual will be temporarily suspended for the given treatment arm.

16.3 Accrual: Fifteen patients with MUC1/HER-2 positive breast cancer with no evidence of disease will be randomized to each one of the 3 treatment schedules. The total number of eligible patients to be accrued will be 45. Patients will be allocated to each treatment schedule using a dynamic allocation procedure that balances the marginal distribution of type of dominant disease between treatment combinations. The expected accrual rate for this study is about 15-20 patients at Mayo Clinic Rochester and about 5-7 patients each at Mayo Clinic Scottsdale and Mayo Clinic Jacksonville per year.

16.4 Study Endpoints:

16.4.1 Primary Endpoints

16.4.1.1 The immunologic parameters of interest are: (1) the percentage of CD4+ T cells, CD8+ T cells, B cells, monocytes, and dendritic cells in a patient's peripheral blood sample as estimated by flow cytometry with a panel of monoclonal antibodies and (2) the frequency of both peptide-specific IFN-gamma producing T cells and peptide-specific IL-5 producing T cells estimated by ELISPOT assays following *in vitro* stimulation with peptide-sensitized stimulator cells for the MUC1 and HER-2 peptides.

16.4.1.2 The number and severity of hematologic and non-hematologic toxicities reported using the NCI-CTC version 3.0 criteria

16.4.2 Secondary Endpoints

16.4.2.1 Disease-free survival is defined as the time from registration to the documentation of a first failure where a failure is the recurrence (REC) of breast cancer or a diagnosis of a second primary cancer (NEWP).

16.4.2.2 Overall survival is defined as the time from registration to death due to any cause.

16.5   Analysis plan for each treatment schedule:

16.5.1 Immunologic Parameters

16.5.1.1   All eligible patients who have completed at least one cycle of treatment are evaluable of the analysis of the immunologic parameters.

16.5.1.2   For each of the immunologic parameters, a plot of the parameter level against time will be constructed such that each patient is represent by a line connecting that patient's data points. These plots will enable visual assessment of patterns of change and variability within a parameter as well as a visual assessment of whether the immunologic parameters peak or fall at similar time points.

16.5.1.3   Also, for each of the immunologic parameters, a plot of the percent change from pre-treatment levels against time will be constructed such that each patient is represent by a line connecting that patient's data points. These plots will enable visual assessment of time trends within a parameter controlling for pretreatment levels.

16.5.2 Adverse Events

16.5.2.1   All eligible patients who received at least one vaccination are evaluable for toxicity.

16.5.2.2   The frequency of those hematologic and non-hematologic toxicities considered at least possibly related to treatment will be tabulated by its severity.

16.5.2.3   Circumstances surrounding any treatment-related death will be reported.

16.6   As this is a pilot study, no formal hypothesis tests comparing treatment schedules are planned. An immunization strategy will be considered for further testing if at least 70% patients treated with that strategy had a $\geq$2-fold increase in the percentage of vaccine-peptide specific CD8+ T cells during the course of treatment, with tolerable toxicity.

16.7   The principal investigator and study statistician will review the study every 3 months to identify potential accrual, toxicity, or endpoint problems. In addition, this study will be monitored by the Cancer Center Data Safety Monitoring Board

16.8   Inclusion of Women and Minorities

This study will be available to all eligible patients, regardless of race or ethnic group. There is no information currently available regarding differential agent effects in subjects defined by gender, race, or ethnicity. The planned analyses will, as always, look for differences in treatment effect based on gender and racial groupings. The sample sizes of this pilot study, however, are not sufficient to provide power for such subset analyses.

To predict the characteristics of patients likely to enroll in this trial we have reviewed registration to (non-North American Breast Cancer Intergroup) NCCTG breast cancer clinical trials by race. This revealed that roughly 3% of patients registered into cancer trials during the past five years could be classified as minorities. This would suggest that only 1 or 2 patients in the study sample are expected to be classified as minorities. This precludes the possibility of a separate subset analysis beyond simple inspection of results for the 1 or 2 minority patients.

17.0 Pathology Considerations:

Central review of HER-2 and MUC1 staining.

18.0 Data Collection Procedures

18.1 Data will be entered into the computer within 2 weeks after each evaluation of the patient. After the patient goes off treatment, follow-up information will be collected and entered as specified in Section 18.2. If patient is still alive after 2 years have elapsed from on-study date, no further follow-up is required.

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ROUGH DRAFT
Not to be used with participants

TITLE: MC0338, MUC1/HER-2/neu Peptide Based Immunotherapeutic
  Vaccines for Breast Adenocarcinomas

IRB #: *pending*

RESEARCHER: Dr. S.N. Markovic and colleagues.

PROTOCOL LAST APPROVED BY INSTITUTIONAL REVIEW BOARD:

THIS FORM APPROVED:

This is an important form. Please read it carefully. It tells you what you need to know about this research study. If you agree to take part in this study, you need to sign this form. Your signature means that you have been told about the study and what the risks are. Your signature on this form also means that you want to take part in this study.

Why is this research study being done?

This study is being done to evaluate three different preparations of a breast cancer vaccine to stimulate anti-cancer (T-cell) immune responses and any side effects associated with these treatments. The breast cancer vaccine (MUC-1/HER-2/neu peptides) will be combined with either one or two immune boosting agents (CpG and GM-CSF) believed to be able to make the vaccine more effective. The vaccine will be given as a shot with Montanide ISA 51, which is oil that is mixed with the vaccine so that the vaccine can be released into the body slowly.

The breast cancer vaccine and one of the immune boosting agents (CpG) have not been approved by the Food and Drug Administration (FDA) for commercial use; however, FDA has permitted their use in this research study. GM-CSF is commercially available for use in clinical practice.

How many people will take part in this research study?

The plan is to have about 45 people take part in this study at Mayo Clinic Rochester, Jacksonville and Scottsdale.

What will happen in this research study?

Before you enter the study, you will have a physical examination and blood tests to make sure that you qualify to take part in this study. About 6 tablespoons of blood will be taken for testing.

If you qualify to take part, you will have blood taken for immunologic testing for the study as well as a skin test (allergy test). You will then be randomly assigned (as in the toss of the dice) to be treated with one of three breast cancer vaccines.

Each treatment will consist of one to two subcutaneous (under the skin) injections of the cancer vaccine. The vaccine will be injected under the skin in areas where there has been no surgery. Usual areas of vaccination include the skin of the upper arms and legs. Treatments will be repeated every 4 weeks for 6 months. Before every treatment, you will be seen by your doctor, examined and about 6 tablespoons of blood will be collected to study the development of the anti-cancer immune response. Skin tests will be done prior to treatments #6 (see table below). Evaluations for the cancer (body scans) will be done if and when your doctor thinks it is necessary.

Pre-Study	- Routine and research blood tests - Cancer evaluation (scans) - Physical examination - Skin test
Treatment #1 (month 1)	Vaccine treatment
Treatment #2 (month 2)	- Research blood test collection - Physical examination - Vaccine treatment
Treatment #3 (month 3)	- Research blood test collection - Physical examination - Cancer evaluation (scans) - Vaccine treatment
Treatment #4 (month 4)	- Research blood test collection - Physical examination - Vaccine treatment
Treatment #5 (month 5)	- Research blood test collection - Physical examination - Vaccine treatment
Treatment #6 (month 6)	- Research blood test collection - Physical examination - Cancer evaluation (scans) - Vaccine treatment - Skin test
Every 3 months after the first three months until 5 years after first treatment	- Physical examination - Routine and research blood tests - Cancer evaluation (scans)

How long will I be in this research study?

You will be treated on the study for 6 months, and you will be seen in follow-up until 5 years after your first treatment.

Are there reasons I might leave this research study early?

Taking part in this research study is your decision. You may decide to stop at any time. You should tell the study doctor if you decide to stop and you will be advised whether any additional tests may need to be done for your safety.

In addition, the investigators or Mayo may stop you from taking part in this study at any time if it is in your best interest, if you do not follow the study rules, or if the study is stopped.

Will any biological sample(s) be stored and used for research in the future?

No. Your samples will be used as described for this study, and then will be destroyed.

What are the risks of this research study?

While you are taking part in this study, you are at risk for the following side effects. You should talk to your study doctor and/or your medical doctor about these side effects. There also may be other side effects that are not known. Other drugs may be given to lessen side effects. Many side effects go away shortly after the treatment is stopped, but in some cases side effects can be serious, long lasting, or may never go away. The side effects can be mild or can lead to death.

You will be closely watched by the study team for any side effects. If side effects happen, the study team will take the necessary steps to treat them. This may include stopping the medication an/or stopping the study.

If any new side effects are found as the study continues, you will be notified.

MUC-1/HER-2/neu, breast cancer vaccine: Possible side effects include skin rash, itching, sweating, headache, muscle aches, joint aches, stomach pain, fatigue, cough, injection site reactions (pain, rash, redness, firmness, warmth, bleeding, ulceration, tenderness to touch, numbness, tingling, itching).

Montanide ISA 51: Possible side effects include skin rash, itching, sweating, headache, muscle aches, joint aches, stomach pain, fatigue, cough, injection site reactions (pain, rash, redness, firmness, warmth, bleeding, ulceration, tenderness to touch, numbness, tingling, itching).

CpG: Possible side effects include skin rash, itching, sweating, headache, muscle aches, joint aches, stomach pain, fatigue, cough, injection site reactions (pain, rash, redness, firmness, warmth, bleeding, ulceration, tenderness to touch, numbness, tingling, itching).

GM-CSF (granulocyte macrophage colony stimulating factor): Possible side effects include skin rash, itching, sweating, headache, muscle aches, joint aches, stomach pain, fatigue, cough, injection site reactions (pain, rash, redness, firmness, warmth, bleeding, ulceration, tenderness to touch, numbness, tingling, itching).

When GM-CSF has been given at higher doses as a daily injection the following side effects have also been reported: diarrhea, general weakness, fever, chills, nausea, vomiting, loss of appetite, headache, pain in the bones, joints and muscles. Most of the symptoms were mild or moderate in severity and were less after taking acetaminophen (Tylenol). Other side effects which happened very rarely were: difficulty breathing, rapid

or irregular heart beat or other heart problems, swelling. Even less common, reported side effects have been the following: 1) increased white cells in the lungs with breathing problems; 2) a syndrome of shortness of breath, low oxygen in the blood, redness in the skin, low blood pressure and dizziness when you stand up or a loss of balance and partial loss of consciousness; 3) serious allergic reactions (like a very severe asthma attack); 4) blood clotting; 5) facial flushing; 6) kidney or liver problems; 7) worsening of fluid accumulation in the arms, legs, lungs or around the heart which may cause problems with breathing or heart failure; 8) patients with heart, lung, kidney or liver problems may have worsening of their symptoms following GM-CSF; and 9) nerve toxicity (weakness, shooting pains, numbness, increased sensitivity to touch, loss of balance, dizziness)

Skin testing: The risks and discomfort of skin testing are minimal and usually limited to bleeding, bruising, or infection at the injection site.

Blood draws: A blood drawing may cause slight pain and a small risk of bleeding, bruising, or infection at the injection site.

There is not enough medical information to know what the risks might be to a breast-fed infant or to an unborn child of a woman who takes part in this study. Women who can become pregnant must use one of these birth control plans during this study: diaphragm, birth control pills, injections, intrauterine device (IUD), surgical sterilization, under the skin implants, abstinence. Another choice is for your sexual partner to use one of these birth control plans. Breast-feeding mothers must stop breast-feeding to take part in this study. Women who can become pregnant must have a pregnancy test before taking part in this study. For the pregnancy test, blood will be taken from a vein in your arm with a needle within 7 days before you enter the study. You will be told the results of the pregnancy test. If the pregnancy test is positive, you will not be able to take part in the study.

Are there benefits to taking part in this research study?

This study may not make your health better. However, the information learned may benefit future patients with breast cancer.

What other choices do I have if I don't take part in this research study?

You do not have to be in this study to receive treatment for your condition. Your other choices may include participation on other clinical studies or no treatment at all, but you will have regular appointments with your doctor who will check your condition. You should talk to your doctor about your choices before you decide if you will take part in this study.

Will I need to pay for the tests and procedures?

The vaccine used in this study will be free of charge.

In the event of side-effects you and/or your health plan will need to pay for all costs associated with the treatment of these side effects. You and/or your health plan may also have to pay for other drugs or treatment that are given to help control side effects as well as the cost of tests or exams to evaluate possible side effects.

You will not need to pay for any tests and exams that are done just for this research study, including research blood tests, skin tests and office visits done only for this research study. However, you and/or your health plan will need to pay for all other tests and exams that you would normally have as part of your regular medical care.

What happens if I am injured because I took part in this research study?

If you have side effects from the study treatment, you need to report them to the researcher and your regular physician, and you will be treated as needed. Mayo will bill you and your insurer for these services at the usual charge. Mayo will not offer free medical care or payment for any bad side effects from taking part in this study. You should discuss this issue thoroughly with the study doctor before you enroll in this study.

If you have out-of-pocket medical expenses for a research-related injury, you may contact the U.S. Army Medical Research and Materiel Command, Office of the Staff Judge Advocate at (301) 619-7663/2221 to request reimbursement. This is because the research tests in this study are partially paid for through a grant with the U.S. Department of Defense. Reimbursement of these expenses cannot be guaranteed, however. You would also be eligible for care in an Army medical treatment facility for the research related injury at no cost to you, if the facility is able to provide the necessary care. For more information about seeking care in an Army medical facility, contact Office of the Staff Judge Advocate at (301) 619-7663/2221. This does not mean that you are giving up any legal rights that you may have.

What are my rights if I take part in this research study?

Taking part in this research study does not take away any other rights or benefits you might have if you did not take part in the study. Taking part in this study does not give you any special privileges. You will not be penalized in any way if you decide not to take part or if you stop after you start the study. Specifically, you do not have to be in this study to receive or continue to receive medical care from Mayo Clinic. If you stop the study you would still receive medical care for your condition although you might not be able to get the study drug.

You will be told of important new findings or any changes in the study or procedures that may affect you or your willingness to continue in the study.

To keep your health information confidential, your address will not be provided in this consent form. However, if the Principal Investigator of this research study learns about new information regarding this research study, he will be able to get your address from your medical record so that he can contact you if necessary.

Who can answer my questions?

You may talk to Dr. Svetomir Markovic at any time about any question you have on this study. You may contact Dr. Markovic (or an associate) by calling the Mayo operator at telephone (507) 284-2511.

You can get more information about Mayo policies, the conduct of this study, or the rights of research participants from the administrator of the Mayo Foundation Office for Human Research Protection, telephone (507) 284-2329 or toll free (866) 273-4681.

Where can I get more information about clinical trials?

You may call the NCI's Cancer Information Service at 1-800-4-CANCER (1-800-422-6237) or TTY: 1-800-332-8615

Visit the NCI's Web sites: Cancer Trials: comprehensive clinical trials information http://cancertrials.nci.nih.gov

CancerNet™: accurate cancer information including PDQ http://cancernet.nci.nih.gov

Authorization To Use And Disclose Protected Health Information

By signing this form, you authorize Mayo Clinic Rochester and the investigators to use and disclose any information created or collected in the course of your participation in this research protocol.

This information may include information relating to sexually transmitted disease, acquired immunodeficiency syndrome (AIDS), or human immunodeficiency virus (HIV). It may also include information relating to behavioral or mental health services or treatment and treatment for substance abuse.

This information may be given to other researchers in this study (including those at other institutions), representatives of the company sponsoring the study, or private, state or federal government parties responsible for overseeing this research. These may include the Food and Drug Administration, the Office for Human Research Protections or other offices within the Department of Health and Human Services, and the Mayo Foundation Office for Human Research Protections or other Mayo groups involved in protecting research subjects. Representatives of the U.S. Army Medical Research and Material Command are eligible to review research records as part of their responsibility to protect human subjects in research.

This information will be given out for the proper monitoring of the study, checking the accuracy of study data, analyzing the study data, and other purposes necessary for the proper conduct and reporting of this study.

This authorization lasts until the end of the study.

You may stop this authorization at any time except if Mayo Clinic Rochester needs information already collected to ensure complete and accurate study results. This might mean that Mayo may continue to use your information collected as part of this study even after you have told us to stop. Since this is a research study that also involves treatment, you may no longer be eligible to receive study treatment if you tell Mayo to stop using this information. The only way you can tell Mayo to stop using the information is in writing addressed as follows:

Mayo Foundation
Office for Human Research Protections
ATTN: Notice of Revocation of Authorization
200 First St. SW
Rochester, MN 55905

If this information is given out to someone else, the information may no longer be protected by federal privacy regulations and may be given out by the person or entity that receives the information.

A copy of this form will be placed in your medical record.

I have had an opportunity to have my questions answered. I have been given a copy of this form. I agree to take part in this research study.

_____   _____   _____
(Date / Time)   (Printed Name of Participant)   (Clinic Number)

                       _____
                       (Signature of Participant)

_____   _____
(Date / Time)   (Printed Name of Individual Obtaining Consent)

                       _____
                       (Signature of Individual Obtaining Consent)

Myung-Joo Patricia Hong
Regulatory Project Manager
Regulatory Management Staff
Office of Cellular, Tissue and Gene Therapies
Center for Biologics Evaluation and Research
Food and Drug Administration
1401 Rockville Pike,
Rockville MD 20852-1448

Dear Ms. Hong,

In response to your letter of April 21, 2004 summarizing our pre-IND teleconference regarding our clinical trial entitled: **"MUC1/HER-2/neu Peptide Based Immunotherapeutic Vaccines for Breast Adenocarcinomas",** please find enclosed:

1) Our responses to your suggestions cited in the letter of April 21, 2004;
2) The revised version of the clinical trial and informed consent document in accordance to your recommendations;
3) Manufacturing documentation and certificates of analysis relating to the production of the peptides used for this study provided to us by the manufacturer (Clinalfa);
4) Letters from the manufacturers of CpG-ODN (Coley Pharmaceutical Group Inc.) and GM-CSF (Berlex Labs) allowing us to cross-reference the master files on these agents for the purpose of this Investigational New Drug application.

Thank you for your consideration of our application.

Sincerely,

Svetomir N. Markovic, M.D., Ph.D.
Associate Professor of Oncology and Medicine
Mayo Clinic

Appendix 1:
Responses to Pre-IND meeting summary letter of 4-21-04

Sponsor question 1:

The manufacturer (Clinalfa) of the peptides that we intend to use in our clinical study has suggested that they apply GMP-like quality standards of manufacturing. Namely, drug substance (API) would not be produced under GMP conditions, but drug product (IMP) will be produced under GMP quality according to ICH guideline Q7A, paragraph 19. According to them, they have already successfully supported prior IND application using this approach. I would ask for your confirmation of this statement.

CBER Response:

Yes.. The product produced under the GMP-like quality standards is acceptable for the use of Phase 1/2 clinical trials

Sponsor question 2:

I would greatly appreciate if you would outline the list of testing requirements of the product that we would need to submit to you prior to approval of the IND. The proposed list of document is as follows:

- Check of bulk material according to Clinalfa's SOP;
- Reconstitution of bulk;
- Sterile filtration;
- Aliquotation;
- Lyophilisation;
- Check of sterility;
- Check of pyrogens (rabbits);
- Abnormal toxicology in two species, 7 day observation (general safety test);
- HPLC check of final vials for identity, purity, quantity;
- Stability testing upon SOP schedule

CBER Response:

1. Regarding the check of bulk materials (lyophilization and aliquotation):

   Please provide detailed description of the bulk materials manufacturing and cleavage process, and reagents/equipment used in the process. Please list names of all reagents used in the manufacturing process, and their certificates of analysis (CoAs). It is recommended that you include the name and address of the provider and species of sources of amino acids in the IND submission. Purification procedure using HPLC should be described in detail. Also, it is recommended that the flow charts and standard operative procedures (SOPs) for quality control be provided.

Sponsor Response: *see attached manufacturing documents (Appendix 3).*

2. Regarding sterile filtration/sterility check (final product):

   In general, lyophilized peptides are reconstituted in sterile distilled deionized water and sterile filtered through a 0.22 μm filter. We recommend that you perform testing for sterility as required by 21 CFR 610.12 and endotoxin test (e.g., a gel-clot limilus amebocyte lysate (LAL) assay; endotoxin levels should be less than 5 EU/mg/kg/hr) as described in CFR to validate the reconstituted products.

Sponsor Response: *No endotoxin test was performed. Sterility testing was done according to current USP guidelines.*

3. Regarding the check of pyrogens (final product), please provide method for pyrogen testing (e.g., kinetic chromogenic and results).

Sponsor Response: *Please see attached reports (Appendix 3).*

4. Regarding general safety test (GST) (final product), the GST is not required at Phase I trials of cellular/gene therapy products and synthetic peptide products.

Sponsor Response: *Please see attached reports (Appendix 3).*

5. Regarding HPLC check of final vials for identity, purity, and quantity:

   - **Identity** – Identity of peptides can be evaluated by mass spectroscopy (molecular weight), amino acid analysis (AAA), sequencing, or any other test. Please include description on appearance, water content, and net peptide content in each vial.
   - **Purity** – Purity of peptides can be analyzed by HPLC (please provide HPLC spec data and % purity) and SDS-PAGE analysis among other tests.
   - **Quantity** – Strength of peptides can be measured by Bradford protein assay.

Sponsor Response:
   - *Identity of the vials has been done by co-elution with drug substance. Drug substance identity has been performed by MS (see CoA of the active ingredient).*
   - *Done by HPLC.*
   - *Performed by HPLC in comparison with standard.*

6. Regarding stability testing of vialed product upon SOP schedule:

   It is recommended that sponsor provides stability testing <u>plan</u> for lot vials with methods and results in tabular form. Testing may be performed at 3, 6, 12, 24, and 36 months time period, and it is recommended that product stability for all three peptides be assessed. We recommend stability testing be performed on material stored at room temperature and/or +4°C storage conditions. Additional tests for stability measure include endotoxin, freedom

from microbial contamination, identity, purity, and appearance. These stability assessments need to be done for all three peptides.

Sponsor Response: *Stability testing will be performed at 6, 12, 24 and 36 months. The test is limited to HPLC purity. IN addition we have performed accelerated stability testing (transport simulation test: vials kept for 7 days at room temperature have been compared by HPLC for purity with vials stored at −18°C).*

CBER provided additional comments regarding the manufacturing process and release testing of the product:

- We recommend that a potency assay be developed to assess the biological function of the product. Please be advised that you will be required to establish a potency assay before initiating Phase 3 trials and validate this assay in support of licensure. Potency assay is a measurement of biological responses (or biological activity) to ensure product quality and lot-to-lot consistency.

- Please include the final lot release data and characterization of your product in the IND submission when available.

- Please describe the transportation and storage conditions of products (e.g., courier, temperature).

- Please provide the labeling information for the lyophilized vaccine product vials.

- Please provide identity testing data on Montanide ISA-51 (Seppic, Inc.).

- Please submit CoAs for the Montanide ISA-51, GM-CSF, and CpG. Letters of authorization to cross-reference INDs utilizing Montanide ISA-51 and CpG-7909 with previous clinical information is required.

Sponsor Response:
- *Not applicable.*
- *Attached (Appendix 3).*
- *Courier at ambient temperature due to the results of the transport simulation data (see above).*
- *See attached label examples (Appendix 3).*
- *See attached (PENDING).*
- *See attached (Appendix 4).*

Clinical

Sponsor question 3:

I would appreciate any comments you might have on the study design of the proposed clinical trial as it pertains to the IND application.

CBER Response:

CBER provided general comments regarding the proposed clinical studies:

1. Regarding proposed trial enrollment/eligibility criteria:

   a. Your protocol eligibility requirements do not limit eligibility by disease stage, except as requiring surgery, chemotherapy, and/or radiation therapy, and in fact need to clarify that the latter treatments refer just to first-line therapy (not so stated in the eligibility section). Risk for recurrence may vary in this heterogeneous group, and it might be beneficial to be more selective even in this early trial. We recommend that to further clarify protocol eligibility you do the following:

   - Please state more explicitly that the permitted prior therapy is first-line only.
   - Please consider limiting the eligibility to more advanced disease stages.

   Sponsor Response: *Section 3.1 of the clinical trial has been modified to include patients with resected stage II or III breast cancer that have completed FIRST LINE standard therapy only. Considering that the goals of this study are immunization efficacy, we elected to proceed with a less advanced breast cancer patient population in order to allow sufficient time for immunizations to become effective. Published data seems to suggest that repeated immunization with class I HLA binding peptides is necessary to generate measurable immune responses. Had we selected a more advanced stage patient population, tumor recurrences may prohibit the completion of a full cycle of immunization. If successful, the follow-up study will target a higher risk population.*

   b. A protocol requirement is that a candidate must be at least 12 months from completing standard therapy (including adjuvant therapy), but an outer time limit is not specified. Also, the protocol does not explicitly state that only one anti-cancer therapy has been given. As written, only investigational cancer therapy is excluded. To minimize the impact of other factors on analysis of results, such as tumor responsiveness to initial treatment or need for other intercurrent treatment, we recommend that you consider adding a statement that a potential candidate may not be on any other anti-cancer therapy at time of trial entry, or concurrently during the experimental therapy, and that you also consider placing an outer time limit on the permissible time prior to enrollment and since completion of standard therapy (e.g., two years or less).

   Sponsor Response: *Section 3.1 of the protocol has been modified to reflect the suggested changes. We are only allowing patients that have completed standard first line therapy for stage II or III breast cancer at least 12 months but no longer than 5 years following last treatment. All patients need to be disease free at time of registration and on no concurrent therapy with the exception of anti-estrogens.*

2. Regarding the proposed therapy:

a. As the trial is designed now a patient may receive up to six product cycles (each is a single product dose on day 1 and day 28 follow-up period). Your proposed doses for Montanide ISA-51 and GM-CSF are somewhat above those studied in previous trials or pre-clinically, and thus are somewhat suspect in terms of what they might add to the toxicity profile anticipated to occur in each of the proposed dosing regimens. Additionally, you have provided no justification for a multiple product dose regimen. We recommend that either you provide more explicit information supporting your plan to administer 6 fixed product doses, or else revise the trial treatment plan to provide complete safety information on a single product dose before proceeding to the sequential dose regimen.

    Sponsor Response: We would prefer to provide the justification for the six doses, and would pre-clinical information as well as available clinical trial information be acceptable?

    CBER Response: Yes.

    Sponsor Response: *Recent publications (Powell, DJ, and Rosenberg SA, J. Immunother. 2004, 27:36-47), ongoing peptide vaccine clinical trials by other groups (e.g. E4697), as well as our own clinical trials data utilizing peptide based vaccines for metastatic melanoma (peptide suspended in Montanide ISA 51 and GM-CSF) demonstrate that **repeated immunization** is necessary for the achievement of detectable anti-peptide vaccine immune responses. In our recently completed MC9973 clinical trial where patients with metastatic melanoma were immunized with three class I HLA binding melanoma differentiation antigen peptides, only 5 of the 27 patients tested demonstrated detectable levels of vaccine specific cytotoxic T lymphocytes (CTL). In each case, the numbers of CTL determined by tetramer-based flow cytometry increased as a result of repeated immunization (data in preparation for publication). The vaccine preparations in this study consisted of peptides co-emulsified with GM-CSF in Montanide ISA 51 (similar to our current proposal).*

b. It is unclear whether all product doses (if repeat dosing cycles do occur) are required to be delivered into the same drainage area for axillary or inguinal nodes given that injection sites are to be rotated per Section 7.3. We recommend that you clarify the protocol text to describe whether, in the event of evaluation of multiple product doses, all such doses will be delivered into rotating sites within the same node drainage area or whether the node drainage areas in turn will be rotated.

    Sponsor Response: The plan is to rotate the injections into different drainage areas. We will clarify the plan.
    Section 7.3 has been modified to reflect the injection rotation scheme that will be employed in this study. We also added an example of injection rotation for a patient that has undergone left axillary lymph node dissection.

c. Your analytic plan addressing response evaluation would evaluate DFS and OS, yet in protocol Section 11.0 patients are subcategorized as REC and NEWP. We are unclear what value this sub-classification has for the current trial proposal or subsequent product development, and recommend that you clarify this in the analytic plan.

Sponsor Response: We understand and will address this in the analytic plan.

Section 11.0 has been modified to clarify the clinical end-points of the study. Disease free survival (DFS) is the time interval from "on-study" registration until breast cancer recurrence (REC) or the development of a new primary (NEWP) cancer. Overall survival (OS) refers to the time interval from "on- study" registration until death from any cause. These definitions are further clarified in section 16.4.2. These represent secondary clinical end points of the current study, and are commonly used for therapeutic clinical trails of adjuvant (post-surgical) therapy of resected breast cancer.

3. Regarding proposed monitoring and analysis:

a. Section 16.4.1.2 of your protocol proposes to grade toxicities according to the new NCI CTC, version 3.0, while elsewhere in the protocol you propose to use version 2.0. We recommend that you adopt version 3.0 as the working toxicity grading scale and eliminate this inconsistency.

b. It is unclear what the safety-monitoring period is for the proposed 6-dose treatment regimen. Long-term follow up according to the table in Section 4.0 is for 2 years, but there is no text that otherwise describes the proposed safety evaluation period. Please consider revising the protocol to describe the safety evaluation period to be analyzed (e.g., one month for acute toxicity and two years for long-term follow up).

c. ADR reporting and plan for product discontinuation due to toxicity (Section 8.0) identifies grade 3 and higher hematologic and grade 4 or higher non-hematologic toxicities as necessitating product dosing discontinuation. We recommend a lower grade cut-off for non-hematologic toxicities. Specifically, we recommend that Section 8.0 be revised to include as dose limiting toxicity (DLT) grade 3 non-hematologic toxicities and to include grade 2 neurologic toxicities that do not resolve.

d. Please clarify the analytic plan as it would pertain to a single-dose trial, or else provide rationale for multiple-dose regime.

Sponsor Response: We will address issues 3a through 3d.

3a. We will utilize NCI-CTC 3.0 throughout the trial.

3b. As suggested, we have included a 4 week "early" safety evaluation period for acute toxicity (see section 4.0) followed by a 2 year follow-up for late toxicities.

3c. Modification of toxicity stopping rules and DLT have been made in accordance to these suggestions (see section 8.0).

3d. The analysis of the clinical trial data has been modified to reflect a multiple-dose regimen by stating that patients eligible for analysis will be those that have completed <u>at least</u> one cycle of immunization (section 16.5). A patient that fails to

complete at least one cycle of immunization will be replaced on the study. In view of our experience with other similar immunization clinical trials, we expect that the majority of patients will complete the full protocol treatment. We did not feel that we could justify replacing patients on the study that have undergone some, but not complete protocol treatment. This is particularly relevant as one of the planned analyses will evaluate the average immunization efficacy based on number of immunizations (e.g. average immunization efficacy as determined by CTL frequencies following one cycle of immunization will be compared to that following two, three, etc, cycles of immunization). If a patient is able to complete 4 of the planned 6 cycles of immunization, their data from the first 4 cycles will still be usable in the analyses.

***CBER provided general comments regarding* the pre-clinical studies:**

4. No detailed information about the pre-clinical studies was provided in the meeting package. Please provide the data from your *in vitro* and *in vivo* experiments or information from the reported literatures to support your proposal for the use of your peptide vaccine and adjuvants.

Sponsor response: *We have expanded the background section of the study to include examples from the literature that are pertinent to our selection of adjuvants for the peptide vaccine (section 1.13). A summary of our preclinical data demonstrating proof-of-principle is enclosed in appendix 1-a.*

5. In your pre-IND meeting package, you stated that the proof-of-principle data was located in appendices 1 & 2. Please provide detailed textual methodology and results including any toxicology data.

Sponsor response: *See above.*

6. Please provide a rationale for your selection of the dose levels of the peptide vaccine and of each adjuvant, the route of administration, and the immunization regimen in support of your proposed clinical trial.

Sponsor response: *The background section of the protocol (section 1.13) has been expanded to include clarification of the stated questions.*

7. Please combine the data from *in vitro* studies that are included in the meeting package (section 1.12, pages 9 to 12) with the above pre-clinical studies in order to facilitate the review.

Sponsor response: *A summary of our proof-of-principle pre-clinical data is enclosed (appendix 1-a).*

FDA Attendees:
Koji Kawakami, Division of Cellular and Gene Therapies, OCTGT

Raj Puri, Division of Cellular and Gene Therapies, OCTGT
David Maybee, Division of Clinical Evaluation & Pharmacology/Toxicology Review, OCTGT
Steven Hirshfeld, Division of Clinical Evaluation & Pharmacology/Toxicology Review, OCTGT
Yongjie Zhou, Division of Clinical Evaluation & Pharmacology/Toxicology Review, OCTGT
Mercedes Serabian, Division of Clinical Evaluation & Pharmacology/Toxicology Review, OCTGT
Myung-Joo Patricia Hong, Regulatory Management Staff, OCTGT

Sponsor Attendees:

Svetomir N. Markovic, M.D., Ph.D., Mayo Clinic
Michelle Daiss, Mayo Clinic

CERTIFICATE OF ANALYSIS for CLINALFA® PRODUCTS

PRODUCT NAME: **HER 2/neu (435-443)**
PRODUCT NUMBER: **C- S-242**   BATCH NUMBER: **AC 0522**

ACTIVE INGREDIENT NAME: H-Ile-Leu-His-Asn-Gly-Ala-Tyr-Ser-Leu-OH (ILHNGAYSL)

ACTIVE INGREDIENT FORMULA: $C_{45} H_{70} N_{12} O_{13}$ MOLECULAR WEIGHT: 987.1

TESTS (Methods)	SPECIFICATIONS	RESULTS at RELEASE 9-Jul-04	at RETEST
IDENTITY of Active Ingredient (coeleution, HPLC/UV)	Single Principal Peak	complies	
VIAL CONTENT of Active Ingredient (mean of N vials, HPLC/UV)	0.90 - 1.10 mg /N=6	1.08 mg /N=6	
UNIFORMITY of Vial Content (mean of N vials, HPLC/UV)	CV ≤ 5 % /N=6 (CV=Coefficient of Variation)	0.5 % /N=6	
ACTIVE INGREDIENT RELATED IMPURITIES (mean of N vials, HPLC/UV)	Total ≤ 5 % /N=6 Any ≤ 3 % /N=6	0.6 % /N=6 complies	
ACTIVE INGREDIENT PURITY (mean of N vials, HPLC/UV)	≥ 95 % /N=6	99.4 % /N=6	
STERILITY (thioglycollate & CASO, EP/USP membrane filtration method)	No evidence of microbial growth	complies	
PYROGENS (body temperature in rabbits, EP/USP method, i.v. dose: 0.03 mg /kg)	Summed response in 3 rabbits ≤ 1.15 °C	complies	

N. D. = NO DATA

NOTES:
Excipients: -
Soluble in 0.9 % NaCl
Vials sealed under N2.
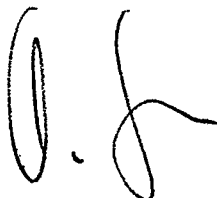
Store at ≤ -18 °C, protect from light.

Estimated Expiry Date (Retest Date):   7-Jan-05

DATE: 16-Jul-04

A. Sauter, PhD
Director Quality Management

To be Used in Approved Clinical Trials Only!
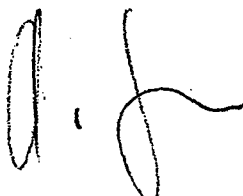
CERTIFICATE OF ANALYSIS for CLINALFA® ACTIVE INGREDIENTS

PRODUCT (API) NAME:	HER 2/neu (435-443)		
API NUMBER: 05-59-0438	API BATCH NUMMER: A 31046		MOL Wt.: 987.1
CHEMICAL NAME: (AA SEQUENCE)	H-Ile-Leu-His-Asn-Gly-Ala-Tyr-Ser-Leu-OH (ILHNGAYSL)		FORMULA: C45 H70 N12 O13

TESTS	SPECIFICATIONS	RESULTS
Appearance	white to off white, amorphous powder	white, amorphous powder
Mass Spectrum	ES-MS: monoisotopic, non-protonated mass = 986.5 ± 1 Da (deduced from series of m/z signals)	986.6 Da
Amino Acids	Ala: 1.0 Asx: 1.0 Gly: 1.0 His: 1.0 Ile: 1.0 Leu: 2.0 Ser: 1.0 Tyr: 1.0 (standard values)	Ala: 1.1 Asx: 1.1 Gly: 1.1 (ADOPTED DATA) His: 1.1 Ile: 1.0 Leu: 2.0 Ser: 1.1 Tyr: 1.1
Purity (HPLC/UV)	Impurities < 2 % Purity > 98 % (product related impurities)	99.8 %
Net Peptide Content	not specified (elementary N-analysis)	89.3 %
Water (GC)	not specified	6.5 %
Acetic Acid	not specified	2.6 %
Trifluoro Acetic Acid	< 1 %	< 0.1 %
Acetonitrile	< 400 ppm	< 30 ppm
INFORMATION	N. D. = NO DATA	Store dry at ≤ -18 °C

DATE: 7-Jun-04

A. Sauter, PhD
Director Quality Management

Merck Biosciences AG
Attn. Dr. H. Märki
Weidenmattweg 4

CH -4448 Läufelfingen

L + S – Nr.: 06559654

Date: 28.06.04
Page 1 of 1

TEST REPORT on the Test for Abnormal Toxicity

Sample	: Her2/neu (435-443)
Batch-No.	: ProduktNr.: 05-59-0438
Designation	:
Date of message: 04.06.2004	Start of examination: 21.06.2004
Arrival : 09.06.2004	End of examination : 28.06.2004

Performance :

- Method: according to USP 27/ Ph. Eur. 4th edition.
- Test solution: The content of one vial was dissolved with 3.5 ml aqua ad iniect.
- Test: 1st test
- Animals: 5 mice/2 guinea-pigs
- Application: i.p. injection
- Dose per mouse: 0.5 ml
- Dose per guinea-pigs: 1.0 ml
- Duration: 7 d

Species	Mice					Guinea-pigs	
Animal No.	1	2	3	4	5	1	2
Weight in g: beginn of test	17.6	18.4	18.5	17.9	17.5	266	274
Weight in g: end of test	18.9	19.9	19.7	19.5	19.2	282	291

Annotation: The content of one vial (2 mg) was dissolved with 3.5 ml water for injection.
Dose per mouse 0.5 ml (pure). Dose per guinea pig 1.0 ml (1:2 with water for
injection diluted).

Result:
All animals survived, no symptoms or body weight reduction were observed

The substance meets the requirements

Dr. Lothar Bomblies

i.V. Dr. Dorothee Jäger
(Head of Department)

L+S AG

Labor L+S AG · Mangelsfeld 4 · D-97708 Bad Bocklet-Großenbrach

Fon +49 (0) 97 08 / 91 00-0 · Fax +49 (0) 97 08 / 91 00-36
e-mail: Labor@Labor-LS.de · Internet: www.Labor-LS.de
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Die Akkreditierung gilt für die in der Urkunde
aufgeführten Verfahren.

Deutscher
Akkreditierungs-
Rat

DAP-PL-2542.00

Merck Biosciences AG
Attn. Mr. A. Sauter
Weidenmattweg 4

CH -4448 Läufelfingen

L + S - No.: 06612784

Date: July 12, 2004 / IT

Page :1 of 1

Test Report on the Test for Pyrogens	
Sample	: HER 2/neu (435-443)
Description	: Batch No.: AC 0522
Reference	: Lieferscheinnr.: 02554
Date of order	: 06-08-2004
Sample receipt	: 06-25-2004

Start of Examination : 06-29-2004
End of Examination : 06-29-2004

Procedure:

Realization: according to USP 27/ Ph. Eur. 4th edition.

Application: as described under "remark"

Injected volume: 1 ml/kg corresponding to a dose of: 0,03 mg/kg

Remark: The content of one Vial was dissolved with 35 ml of 0.9% sodium chloride solution.

Test: first Test

Measuring:

Animal No.	Temp. in °C before Injection				Injec-tion	Temp. in °C after Injection					
	90'	60'	30'	0'		30'	60'	90'	120'	150'	180'
1	39,2	39,2	39,2	39,1	tion	39,1	39,1	39,1	39,1	39,1	39,1
2	39,4	39,4	39,4	39,3		39,3	39,3	39,3	39,3	39,3	39,2
3	39,3	39,3	39,2	39,2		39,2	39,2	39,3	39,3	39,3	39,2

Evaluation:

Animal No.	Initial Temp. in °C (Mean of 30' and 0')	Maximum Temp. in °C (after Injection)	Difference in °C
1	39,15	39,1	0,00
2	39,35	39,3	0,00
3	39,20	39,3	0,10
		Sum:	0,10

Result/Conclusion:

The sample passes the test for pyrogens.

Instead of a sham test according to USP 27, a preliminary test according to
Ph. Eur. 4th edition was done.

Dr. Lothar Bomblies

i. V. Dr. Jürgen Balles (Abteilungsleitung)

The test results apply solely to the designated sample.
Reproduction in the form of an extract may only be made with the written consent of Labor L+S AG.

LSAG

Merck Biosciences AG
Attn. Mr. A. Sauter
Weidenmattweg 4

CH -4448 Läufelfingen

L + S - Nr.: 06612784

Date: July 14, 2004 / UM

Page :1 of 1

Test Report on the Test for Sterility

Sample	: HER 2/neu (435-443)
Description	: Batch No.: AC 0522
Reference	: Lieferscheinnr.: 02554
Date of order	: 06-08-2004
Sample receipt	: 06-25-2004

Start of Examination: 06-25-2004
End of Examination : 07-09-2004

Procedure:

- Number of samples tested: 20
- Membrane filtration according to Ph. Eur. 4th Edition/ USP 27
- Samples tested pooled
- Media used: Thioglycolat and CASO
- Test systems used: TTHA DV (Millipore)
- Incubation time: 14 days
- Special procedures: Dissolving and neutralization of the sample
 with peptone buffer.

Result / Conclusion

Sterile

The result complies with the requirements of the Test for Sterility

Remark: Growth control according to L+S SOP was performed.

The results comply with the requirements.

ppa. Dr. Frank Böttcher

i.V. Dr. Timo Krebsbach (Abteilungsleitung)

Synthesis and Purification Protocol for Her2/neu (435-443) C-S-242

Product Number APC: 322157
Lot Number APC: S05024A1
Sequence: **Ile-Leu-His-Asn-Gly-Ala-Tyr-Ser-Leu**

The peptide was synthesized on Wang resin via Fmoc chemistry (see the attachment for details). Protecting groups used for amino acids are: t-Butyl group for Ser and Tyr; Trityl group for Asn and His. Fmoc protected amino acids were purchased from EMD Biosciences and Senn Chemicals USA. Reagents for coupling and cleavage were purchase from Aldrich. Solvents were purchased from Fish Scientific. The peptide chain was assembled on resin by repetitive removal of the Fmoc protecting group and coupling of protected amino acid. DIC and HOBt were used as coupling reagent and N-methylmorpholine was used as base. After removal of last Fmoc protecting group, resin was treated with TFA/TIS/H2O (95:3:2 v/v) cocktail for cleavage and removal of the side chain protecting groups. Crude peptide was precipitated from cold ether and collected by filtration. Purification of crude peptide was achieved via RP-HPLC using 47mm x 300mm column from Waters. Peptide was purified using triethyl phosphate buffer (buffer A) and acetonitrile (buffer B). A linear gradient of 5% to 35% buffer B in 60 minutes was used. Pooled fractions were desalted using acetic acid buffer. The peptide has been verified by MS analysis and amino acid analysis. The peptide purity was determined by analytical HPLC column (C18, 4.6 x 250mm) which was obtained from Supelco.

List of chemicals and solvents used in the synthesis
NMM (N-methylmorpholine)
DIC (Diisopropylcarbodiimide)
HOBt (1-Hydroxybenzotriazole)
Piperidine
TIS (Diisopropylsilane)
DMF (Dimethylformamide)
Diethylether
Trifluoroacetice acid
DCM (Dichloromethane)
Methanol

Attachment

Synthesis Procedure for Peptide

Product Number: 322157

Lot Number: S05024A1
Sequence: Ile-Leu-His-Asn-Gly-Ala-Tyr-Ser-Leu

Step 1 (Resin swelling)

Fmoc-Leu-Wang resin was swelled in DCM for 30 minutes (10 ml/g resin)

Step 2 (Deprotection)

a. Add 20% piperidine/DMF solution (10 ml/g resin) to the resin.
b. Stir for 30 minutes (start timing when all the resin is free floating in the reaction vessel).
c. Drain.

Step 3 (Washing)

a. Wash the resin with DMF (10 ml/g resin) five times. Ninhydrin test: positive.

Step 4 (Coupling)

Fmoc-AA-OH: 3 equivalent relative to resin loading
HOBt: 3 equivalent relative to resin loading
DIC: 3 equivalent relative to resin loading
NMM: 6 equivalent relative to resin loading

Weigh Fmoc-AA-OH and HOBt into a plastic bottle. Dissolve the solids with DMF (5 ml/g resin). Add DIC to the mixture, followed by the addition of NMM . Add the mixture to the resin. Bubble (or stir) gently for 10 – 60 minutes until a negative ninhydrin test on a small sample of resin is obtained.

Step 5 (Washing)

Wash the resin with DMF (3 times).

Step 6

a.   Repeat steps 2-5 until required peptide is assembled.

Step 7 (N-terminal Fmoc de-protection)

a. Repeat step 2 and go to step 8.

Step 8 (Washing and Drying)

a.   After the final coupling, wash resin with DMF (3 times), MeOH (1 time), DCM (3 times) and MeOH (2 times).
b.   Dry the resin under vacuum (water aspirator) for 2 hours and high vacuum (oil pump) for a minimum of 12 hours.

Cleavage

a.   Place dry resin in a plastic bottle and add the cleavage cocktail. Shake the mixture at room temperature for 2.5 hours.
b. Remove the resin by filtration under reduced pressure. Wash the resin twice with TFA. Combine filtrates, and add an 8-10 fold volume of cold ether to get precipitate.
c. Crude peptide was isolated by filtration. Wash the crude peptide with cold ether (twice).

AMERICAN PEPTIDE

HER 2 /neu (435-443)

ILHNGAYSL

June 11, 2004

To: whom it may concern
From: American Peptide Company, Inc.

RE: Statement of Origin of Raw Materials

We, American Peptide Co, hereby confirmed that we purchased all the Fmoc protected L-amino acids used in the production of peptide **322157** (Lot Number: S05024A1) from EMD Bioscience, Inc and Senn Chemicals USA. According to our vendor's statement, all the L-amino acids are of synthetic or plant origin.

Baosheng Liu

Baosheng Liu, Ph.D
Vice President of R&D and Manufacturing
American Peptide Company, Inc.
Tel: 408-733-7604
Fax: 408-733-9057
e-mail: Baosheng@americanpeptide.com

CERTIFICATE OF ANALYSIS for CLINALFA® PRODUCTS

PRODUCT NAME: **HER 2/neu (883-899)**

PRODUCT NUMBER: C- S-243 **BATCH NUMBER: AC 0523**

ACTIVE INGREDIENT NAME: H-Lys-Val-Pro-Ile-Lys-Trp-Met-Ala-Leu-Glu-Ser-Ile-Leu-Arg-Arg-Arg-Phe-OH (KVPIKWMALESILRRRF)

ACTIVE INGREDIENT FORMULA: $C_{100} H_{167} N_{29} O_{21} S_1$ **MOLECULAR WEIGHT: 2143.7**

TESTS (Methods)	SPECIFICATIONS	RESULTS at RELEASE 6-Aug-04	at RETEST
IDENTITY of Active Ingredient (coeleution, HPLC/UV)	Single Principal Peak	complies	
VIAL CONTENT of Active Ingredient (mean of N vials, HPLC/UV)	0.9 - 1.1 mg /N=6	1.0 mg /N=6	
UNIFORMITY of Vial Content (mean of N vials, HPLC/UV)	CV ≤ 5 % /N=6 (CV=Coefficient of Variation)	1.9 % /N=6	
ACTIVE INGREDIENT RELATED IMPURITIES (mean of N vials, HPLC/UV)	Total ≤ 5 % /N=6 Any ≤ 3 % /N=6	0.4 % /N=6 complies	
ACTIVE INGREDIENT PURITY (mean of N vials, HPLC/UV)	≥ 95 % /N=6	99.6 % /N=6	
STERILITY (thioglycollate & CASO, EP/USP membrane filtration method)	No evidence of microbial growth	complies	
PYROGENS (body temperature in rabbits, EP/USP method, i.v. dose: 0.03 mg /kg)	Summed response in 3 rabbits ≤ 1.15 °C	complies	

N. D. = NO DATA

NOTES:
Excipients: -
Soluble in 0.9 % NaCl
Vials sealed under N2.

Store at ≤ -18 °C, protect from light.

Estimated Expiry Date (Retest Date): 4-Feb-05

DATE: 6-Aug-04

To be Used in Approved Clinical Trials Only!

A. Sauter, PhD
Director Quality Management

CERTIFICATE OF ANALYSIS for CLINALFA® ACTIVE INGREDIENTS

PRODUCT (API) NAME:	HER 2/neu (883-899)		
API NUMBER: 05-59-0439	API BATCH NUMMER: A 31076		MOL Wt.: 2143.7
CHEMICAL NAME: (AA SEQUENCE)	H-Lys-Val-Pro-Ile-Lys-Trp-Met-Ala-Leu-Glu-Ser-Ile-Leu-Arg-Arg-Arg-Phe-OH (KVPIKWMALESILRRRF)		FORMULA: C100 H167 N29 O21 S1

TESTS	SPECIFICATIONS	RESULTS
Appearance	white to off white, amorphous powder	white, amorphous powder
Mass Spectrum	ES-MS: monoisotopic, non-protonated mass = 2142.3 ± 1 Da (deduced from series of m/z signals)	2142.3 Da
Amino Acids	Ala: 1.0 Arg: 3.0 Glx: 1.0 Ile: 2.0 Leu: 2.0 Lys: 2.0 Met: 1.0 Phe: 1.0 Pro: 1.0 Ser: 1.0 Trp: 1.0 Val: 1.0 (standard values)	Ala: 1.1 Arg: 3.3 Glx: 1.1 (ADOPTED DATA) Ile: 1.7 Leu: 2.0 Lys: 2.0 Met: 1.1 Phe: 1.0 Pro: 1.0 Ser: 1.0 Trp: *) Val: 1.0
Purity (HPLC/UV)	Impurities < 2 % Purity > 98 % (product related impurities)	99.7 %
Net Peptide Content	not specified (elementary N-analysis)	83.4 %
Water (GC)	not specified	5.2 %
Acetic Acid	not specified	5.4 %
Trifluoro Acetic Acid	< 1 %	**)1.1 %
Acetonitrile	< 400 ppm	<28 ppm
Isopropanol	< 400 ppm	<19 ppm
INFORMATION		Store dry at ≤ -18 °C
	N. D. = NO DATA *) Trp was completely destroyed during hydrolysis **) meets specification of 1% (not 1.0%) when rounded	

DATE: 21-Jun-04

v. R. flour

A. Sauter, PhD
Director Quality Management

LSAG

Merck Biosciences AG
Attn. Dr. H. Märki
Weidenmattweg 4

CH -4448 Läufelfingen

L + S – Nr.: 06559664

Date: 28.06.04
Page 1 of 1

TEST REPORT on the Test for Abnormal Toxicity

Sample	: Her2/neu (883-899)
Batch-No.	: ProduktNr.: 05-59-0439
Designation	:
Date of message	: 04.06.2004	Start of examination: 21.06.2004
Arrival	: 09.06.2004	End of examination : 28.06.2004

Performance :

- Method:	according to USP 27/ Ph. Eur. 4th edition.
- Test solution:	The content of one vial was dissolved with 3.5 ml aqua ad iniect.
- Test:	1st test
- Animals:	5 mice/2 guinea-pigs
- Application:	i.p. injection
- Dose per mouse:	0.5 ml
- Dose per guinea-pigs:	1.0 ml
- Duration:	7 d

Species	Mice					Guinea-pigs	
Animal No.	1	2	3	4	5	1	2
Weight in g: beginn of test	17.9	18.5	17.6	18.1	18.5	261	274
Weight in g: end of test	19.1	20.2	19.3	19.5	20.3	270	282

Annotation: The content of one vial (2 mg) was dissolved with 3.5 ml water for injection.
Dose per mouse 0.5 ml (pure). Dose per guinea pig 1.0 ml (1:2 with water for injection diluted).

Result:
All animals survived, no symptoms or body weight reduction were observed

The substance meets the requirements

Dr. Lothar Bomblies

i.V. Dr. Dorothee Jäger
(Head of Department)

Labor L+S AG · Mangelsfeld 4 · D-97708 Bad Bocklet-Großenbrach

Fon +49 (0)97 08/91 00-0 · Fax +49 (0)97 08/91 00-36
e-mail: Labor@Labor-LS.de · Internet: www.Labor-LS.de
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Deutsches Akkreditierungssystem Prüfwesen GmbH
akkreditiertes Prüflaboratorium.
Die Akkreditierung gilt für die in der Urkunde
aufgeführten Verfahren.

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DAP-PL-2542.00

Merck Biosciences AG
Attn. Mr. A. Sauter
Weidenmattweg 4

CH -4448 Läufelfingen

L + S - Nr.: 07700534

Date: July 29, 2004 / UM

Page :1 of 1

Test Report on the Test for Sterility

Sample	: HER 2/neu (883-899)	
Description	: Lot: AC 0523, BAG-Ch.-B.: 429602	
Reference	:	
Date of order	: 07-13-2004	Start of Examination: 07-15-2004
Sample receipt	: 07-15-2004	End of Examination : 07-29-2004

Procedure:

- Number of samples tested: 20
- Membrane filtration according to Ph. Eur., 4th Edition/USP27
- Samples tested pooled
- Media used: Thioglycolat and CASO
- Test systems used: TTHA DV (Millipore)
- Incubation time: 14 days
- Special procedures: Dissolving and neutralization of the sample
  with peptone buffer.

Result / Conclusion

Sterile

The result complies with the requirements of the Test for Sterility

ppa. Dr. Frank Böttcher

i.V. Dr. Timo Krebsbach (Abteilungsleitung)

The test results apply solely to the designated sample.
Reproduction in the form of an extract may only be made with the written consent of Labor L+S AG.

Merck Biosciences AG
Attn. Mr. A. Sauter
Weidenmattweg 4

CH -4448 Läufelfingen

L + S - No.: 07700534

Date: July 29, 2004 / UM

Page :1 of 1

Test Report on the Test for Pyrogens

Sample	: HER 2/neu (883-899)
Description	: Lot: AC 0523, BAG-Ch.-B.: 429602
Reference	:
Date of order	: 07-13-2004
Sample receipt	: 07-15-2004

Start of Examination : 07-22-2004
End of Examination : 07-22-2004

Procedure:

Realization: according to USP 27/ Ph. Eur. 4th edition.

Application: as described under "remark"

Injected volume: 1 ml/kg corresponding to a dose of: 0.03 mg/kg

Remark: The content of one Vial was dissolved with 35 ml of 0.9% sodium chloride solution.

Test: first Test

Measuring:

Animal No.	Temp. in °C before Injection				Injec-tion	Temp. in °C after Injection					
	90'	60'	30'	0'		30'	60'	90'	120'	150'	180'
1	39,2	39,3	39,2	39,2		39,2	39,2	39,2	39,2	39,1	39,1
2	39,3	39,3	39,2	39,2		39,1	39,1	39,1	39,1	39,1	39,0
3	39,6	39,6	39,6	39,5		39,5	39,5	39,4	39,4	39,3	39,2

Evaluation:

Animal No.	Initial Temp. in °C (Mean of 30' and 0')	Maximum Temp. in °C (after Injection)	Difference in °C
1	39,20	39,2	0,00
2	39,20	39,1	0,00
3	39,55	39,5	0,00
		Sum:	0,00

Result/Conclusion:

The sample passes the test for pyrogens.

Instead of a sham test according to USP 27, a preliminary test according to
Ph. Eur. 4th edition was done.

_____
Dr. Lothar Bomblies

i. V. Dr. Dorothee Jäger (Abteilungsleitung)

Synthesis and Purification Protocol for Her2/neu (883-889) C-S-243

Product Number APC:	311901
Lot Number APC:	S05082A1
Sequence:	Lys-Val-Pro-Ile-Lys-Trp-Met-Ala-Leu-Glu-Ser-Ile-Leu-Arg-Arg-Arg-Phe

The peptide was synthesized on Wang resin via Fmoc chemistry (see the attachment for details). Protecting groups used for amino acids are: t-Butyl group for Glu and Ser; Pbf for Arg; Boc for Lys and Trp. Fmoc protected amino acids were purchased from EMD Biosciences and Senn Chemicals USA. Reagents for coupling and cleavage were purchase from Aldrich. Solvents were purchased from Fish Scientific. The peptide chain was assembled on resin by repetitive removal of the Fmoc protecting group and coupling of protected amino acid. HBTU and HOBt were used as coupling reagent and N-methylmorpholine was used as base. After removal of last Fmoc protecting group, resin was treated with TFA/Thioanisole/Phenol/H_2O/EDT (87.5:5:2.5:2.5:2.5v/v) cocktail for cleavage and removal of the side chain protecting groups. Crude peptide was precipitated from cold ether and collected by filtration. Purification of crude peptide was achieved via RP-HPLC using 47mm x 300mm column from Waters. Peptide was first purified using triethyl phosphate buffer (buffer A) and acetonitrile (buffer B). A linear gradient of 18% to 50% buffer B in 60 minutes was used. Pooled fractions were then purified using TFA buffer (Buffer A: 0.1% TFA in H_2O, Buffer B: acetonitrile). A linear gradient of 15% to 55% buffer B in 60 minutes was used. Pooled fractions were desalted using acetic acid buffer. The peptide has been verified by MS analysis and amino acid analysis. The peptide purity was determined by analytical HPLC column (C18, 4.6 x 250mm) which was obtained from Supelco.

List of chemicals and solvents used in the synthesis

NMM (N-methylmorpholine)
HBTU (2-(1H-Benzotriazole-1-yl)-1,1,3,3-tetramethyluronium Hexafluorophosphate)
HOBt (1-Hydroxybenzotriazole)
Piperidine
Thioanisole
Phenol
EDT (1,2-Ethanedithiol)
DMF (Dimethylformamide)
Diethylether
Trifluoroacetice acid
DCM (Dichloromethane)
Methanol

AMERICAN PEPTIDE

Attachment

Synthesis Procedure for Peptide
Product Number: 311901
Lot Number: S05082A1
Sequence: Lys-Val-Pro-Ile-Lys-Trp-Met-Ala-Leu-Glu-Ser-Ile-Leu-Arg-Arg-
Arg-Phe

Step 1 (Resin swelling)

Fmoc-Phe-Wang resin was swelled in DCM for 30 minutes (10 ml/g resin)

Step 2 (Deprotection)

a.   Add 20% piperidine/DMF solution (10 ml/g resin) to the resin.
b.   Stir for 30 minutes (start timing when all the resin is free floating in the reaction vessel).
c.   Drain.

Step 3 (Washing)

a. Wash the resin with DMF (10 ml/g resin) five times. Ninhydrin test: positive.

Step 4 (Coupling)

Fmoc-AA-OH: 3 equivalent relative to resin loading
HOBt: 3 equivalent relative to resin loading
HBTU: 3 equivalent relative to resin loading
NMM: 6 equivalent relative to resin loading

Weigh Fmoc-AA-OH and HOBt into a plastic bottle. Dissolve the solids with DMF (5 ml/g resin). Add HBTU to the mixture, followed by the addition of NMM . Add the mixture to the resin. Bubble (or stir) gently for 10 – 60 minutes until a negative ninhydrin test on a small sample of resin is obtained.

Step 5 (Washing)

Wash the resin with DMF (3 times).

Step 6

a.   Repeat steps 2-5 until required peptide is assembled.

Step 7 (N-terminal Fmoc de-protection)

a.   Repeat step 2 and go to step 8.

Step 8 (Washing and Drying)

a.   After the final coupling, wash resin with DMF (3 times), MeOH (1 time), DCM (3 times) and MeOH (2 times).

b.   Dry the resin under vacuum (water aspirator) for 2 hours and high vacuum (oil pump) for a minimum of 12 hours.

Cleavage

a.   Place dry resin in a plastic bottle and add the cleavage cocktail. Shake the mixture at room temperature for 2.5 hours.

b.   Remove the resin by filtration under reduced pressure. Wash the resin twice with TFA. Combine filtrates, and add an 8-10 fold volume of cold ether to get precipitate.

c.   Crude peptide was isolated by filtration. Wash the crude peptide with cold ether (twice).

AMERICAN PEPTIDE

HER2/neu (883-899)
KVPIKWMALESIL RRF

June 11, 2004

To: whom it may concern
From: American Peptide Company, Inc.

RE: Statement of Origin of Raw Materials

We, American Peptide Co, hereby confirmed that we purchased all the Fmoc protected L-amino acids used in the production of peptide **311901** (Lot Number: S05082A1) from EMD Bioscience, Inc and Senn Chemicals USA. According to our vendor's statement, all the L-amino acids are of synthetic or plant origin.

Baosheng Liu

Baosheng Liu, Ph.D
Vice President of R&D and Manufacturing
American Peptide Company, Inc.
Tel: 408-733-7604
Fax: 408-733-9057
e-mail: Baosheng@americanpeptide.com

C-S-242   AC0522

HER 2/ neu (435-443)   1.08 mg

ILHNGAYSL

Expiry Date: see Certificate of Analysis   Store at ≤ -18 °C

Merck Biosciences AG
CH-4448 Läufelfingen
Tel. +41 62 285 25 50

CLINALFA

TO BE USED IN APPROVED
CLINICAL TRIALS ONLY

C-S-243   AC0523

HER 2/ neu (883-899)

KVPIKWMALESILRRRF   1.0 mg

Expiry Date: see Certificate of Analysis
Manufacturing Date: 12 Jul 04   Store at ≤ -18 °C

Merck Biosciences AG
CH-4448 Läufelfingen
Tel. +41 62 285 25 50

CLINALFA

TO BE USED IN APPROVED
CLINICAL TRIALS ONLY

COLEY

January 5, 2004

Department of Health and Human Services
Food and Drug Administration
Office of Drug Evaluation 1
Division of Oncology Drug Products (HFD-150)
1451 Rockville Pike
Rockville, MD 20857

Attn: Richard Pazdur, M.D.
        Director
        Division of Oncology Drug Products

Re: Letter of Authorization
        IND #59,429 (CPG 7909 Injection)

For: Svetomir Markovic, MD, Ph.D.
        Division of Hematology/Department of Internal Medicine
        Mayo Clinic Cancer Center
        200 First Street, SW
        Rochester, MN 55905

Dear Dr. Pazdur:

The purpose of this letter is to authorize the Food and Drug Administration to cross-refer information contained in the above-referenced IND, specifically as it relates to Chemistry, Manufacturing and Controls information, filed by Coley Pharmaceutical Group, Inc. into a physician IND to be filed by Dr. Svetomir Markovic, on behalf of the Mayo Clinic Cancer Center.

The CPG 7909 clinical supplies which will be used in the study to be conducted under Dr. Markovic's IND will be provided for under reference to a CMC amendment dated April 16, 2002 (Serial Number 037) relating to CPG 7909 drug substance and a CMC amendment dated December 27, 2001 (Serial No. 031) relating to CPG 7909 drug product.

If you have any questions or require any additional information please contact me at (781) 431- 9000 (ext 1402).

Sincerely,

Deborah DeMuria, Pharm.D.
Director, Regulatory Affairs

cc: Dr. Svetomir Markovic, Mayo Clinic Cancer Center